CHARACTERIZATION OF COMMON CARP (*Cyprinus carpio*) INSULIN-LIKE GROWTH FACTOR GENES

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ABSTRACT

To examine the phylogeny, biochemistry and molecular biology of insulin-like growth factor (IGF) in local fish species, we started our studies using a local tropical fish, the common carp (*Cyprinus caripo*). We have demonstrated that IGF-I Ea2 form is the predominantly expressed form of IGF in common carp.

In the present study, common carp was used to study the molecular biology of IGFs with a view to elucidate their genomic structure and expression in liver in response to growth hormone (GH). The promoter of the common carp IGF-I gene was cloned from carp genomic DNA using polymerase chain reaction (PCR) and this clone carries an insert of 840 basepair encompassing the 5'-UTR and part of the signal peptide. Ten IGF-I gene related clones from a common carp genomic library were isolated after screening of 600,000 plaques. One clone carries an insert of ~13 kb and probably contains the entire common carp IGF-I gene. Using reverse transcription-PCR, the hepatic IGF-I mRNA level was found to be elevated after i.p. injection of GH at a dose of 100 ng/g body weight. With an aim to search for IGF-II, trout IGF-II cDNA was used as a probe to investigate the common carp genomic DNA but no IGF-II gene were identified in the common carp genomic DNA using Southern blot analysis.

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CHAPTER 1 INTRODUCTION

1.1 General Introduction

Growth is one of the fundamental characteristics of all living organisms. A large number of peptide growth factors contribute to the stimulation and regulation of growth. Like other multifunctional growth factors, insulin-like growth factors I and II (IGF-I and IGF-II) elicit diverse effects on a variety of biological processes and have a broad range of functions on the embryo, fetus, and adult. The action of insulin-like growth factors (IGFs) is initiated upon binding to cell-surface receptors and is modulated through interactions with secreted IGF binding proteins (IGFBPs).

The term IGF was proposed to denote peptides which are structurally similar to proinsulin (e.g. 50% amino acid sequence homology) but functionally distinct from proinsulin. The significant differences between these two molecules are: 1) insulin levels fluctuate widely in response to variations in carbohydrate intake; 2) the circulating IGFs bound to carrier proteins with stable plasma concentrations; 3) the insulin and IGFs posses structurally distinct receptors; 4) insulin stimulates glucose transport and metabolic processes, such as glycogen and lipid synthesis, whereas IGFs stimulate cellular replication (Clemmons, 1989). These functional and structural distinctions appear to be very important for the maintenance of normal carbohydrate homeostasis and long-term growth.

It is believed that IGFs are members of a evolutionarily insulin-related gene family which includes insulin, IGF-I, IGF-II, relaxin, insect prothoracicotrophic hormone, and molluscan insulin-related peptide (Blundell and Humbel, 1980; Smit *et al.*, 1989). The prevailing theory on the evolution of this family suggests that an "early" insulin-like gene duplication gave rise to the "primary" family members

including modern insulin and IGF-I, and that IGF-I and IGF-II diverged during the evolution of mammals (Froesch *et al.*, 1985). However, other taxonomically more extensive studies detected IGF-I and IGF-II in birds, reptiles, amphibians, and fish, leading to the proposition that the gene duplication event leading to two IGFs occurred early in vertebrate evolution (Shamblott and Chen, 1992; Reinecke *et al.*, 1995).

Although IGFs have been identified, at either the protein or nucleic acid level, in a number of non-mammalian vertebrates including chicken (Darling and Brickell, 1996), *Xenopus* (Reinecke *et al.*, 1995), rainbow trout (Shamblott and Chen, 1992), and seabream (Duguay *et al.*, 1996), most studies directed at the identification of IGFs and the elucidation of their biological roles have been restricted to mammalian models such as the rat, mouse, and human. As a result of these studies, several generalities can be concluded regarding mammalian IGF structure and biological roles. (1) Two distinct IGF mRNAs resulting from transcription of separate and unlinked genes are present. (2) IGF-I is primarily produced in the liver under the regulation of growth hormone (GH) and tends to increase in level from birth to puberty. (3) IGF-II is primarily produced in the liver under the regulation of placental lactogen during embryonic and neonatal stages of development. (4) Transcription of both IGFs can be detected in a wide variety of cell and tissue types. (5) IGF-I and IGF-II undergo proteolytic cleavage to generate their mature peptides. (6) The IGF-I gene is subject to differential RNA processing to yield two distinct mRNA types but apparently only one mature peptide form. (7) Nutritional status, or the supply of dietary energy and proteins are important regulators of IGFs. IGFs appear to provide an important mechanism linking nutrition and growth (Humbel, 1990; Ketelslegers *et al.*, 1995).

Although significant accomplishments have been made in the genetics and biology of mammalian IGFs, the fact that both the greatest diversity and biomass of life on earth is not represented by mammals suggests there is a great deal to be learned about all facets of biology, including the regulatory processes of growth, from non-mammalian models. Until recently, IGFs have been identified and characterized in a limited number of nonmammalian species. Since both IGF-I and IGF-II mRNA are found in adult and juvenile fish species, it was suggested that both IGF-I and IGF-II may have growth effects in teleost (Shamblott and Chen, 1992; Duguay *et al.*, 1996). However, most of the studies are focused on the salmonids species, like salmon and trout, which live in temperate water with slow growth rate. Whether this phenomenon is universally true for other fish species, particularly those in tropical waters, remains to be established. In order to expand what is known about fish IGFs, we use common carp (*Cyprinus carpio*) as a tropical water fish model to study the molecular biology of fish IGFs.

1.2 Historical Overview

1.2.1 Insulin-Like Growth Factors I and II

Studies on IGF were started in three separate areas of biomedical research: (1) investigation into the actions of GH on growth, (2) studies on the insulin-like effects of components of serum, and (3) assessment of the role of locally secreted factors on cell replication (Froesch *et al.*, 1963; Daughaday *et al.*, 1972; Pierson and Temin, 1972).

Increasing the width of the epiphysial cartilage plate of the tibia has long been used as an assay for GH. In 1957, Salmon and Daughaday found that the effects of GH on cartilage growth in rat occurred though a serum factor, initially designated as

"sulfation factor" because their assay monitored incorporation of radiolabeled sulfate into the extracellular matrix. Incorporation of [^{35}S]sulfate into cartilage is markedly suppressed in hypophysectomized rats and can be stored to normal level within 24 hours by the administration of GH *in vivo*. However, the incorporation of [^{35}S]sulfate into cartilage could not be corrected by addition of GH to media containing cartilage segments *in vitro*. It was found that the addition of diluted normal rat serum but not from hypophysetomized rat was able to stimulate increase in sulfate uptake of the cartilage *in vitro* (Salmon and Daughaday, 1957). These experiments showed that there must be some intermediary substance(s) mediating the action of GH. This sulfation factor which was subsequently termed "somatomedin C" (Daughaday *et al.*, 1972), was ultimately shown to be IGF-I. This hypothesis, commonly referred to as the somatomedin hypothesis, has been extensively confirmed and recently extended to include autocrine and paracrine roles of somatomedins in many tissues as well as the originally recognized endocrine role of this growth factor.

In the 1960s, several laboratories sought to identify components in serum with insulin-like effects on metabolism that were not neutralized by anti-insulin antibodies (Froesch *et al.*, 1963; Megyesi *et al.*, 1974). These substance became known as nonsuppressible insulin-like activity and ultimately were shown to include both IGF-I and IGF-II.

In the early 1970s, Pierson and Temin observed that cultured liver cells could secrete their own "multiplication-stimulating activity" that enhance cell replication (Pierson and Temin, 1972). This factor was later identified as IGF-II (Rinderknecht and Humbel, 1978b).

Initial attempts to purify these factors suggested that they had overlapping activities. With the purification and sequencing of human IGF-I and IGF-II in the late

1970s, two peptides containing three disulfide bridges, both with relative molecular mass (M_r) of 7,500 were identified. It was found that IGF-I was a single-chain basic protein of 70 amino acids and that IGF-II was a slightly acidic single-chain peptide of 67 residues (Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel, 1978b). The amino acid sequences of IGF-I and -II are ~70% identical to one another, but their A and B domains are only ~50% identical to the A and B domains of human insulin.

Comparsion of the primary sequences of IGFs with insulin allows recognition of peptide domains B, C, A, and D, in which domains A and B are structural homology to the insulin A and B chains. Domain C is analogous to the connecting (C) peptide in proinsulin, whereas the D domain is not found in insulin. Both IGFs are synthesized as precursors composed of a signal peptide, the mature peptide, and a trailer E domain, in which the signal peptide and E domain are removed during posttranslational processing (Fig. 1.1). Although neither the physiological significance or the universality of E domain proteolytic cleavage has been established, roles in peptide processing, transport, secretion, degradation, receptor interactions, or binding protein interactions have been postulated (Lowe, 1996).

1.2.2 IGF Receptors

With the availability of highly purified IGFs in the mid 1970s, researchers performed cell surface binding assays to examine whether these ligands interacted with the insulin receptor (Marshall *et al.*, 1974). It was soon shown that iodinated IGF-I bound to cell surface receptor that was distinct from the insulin receptor (Megyesi *et al.*, 1975). In 1980, the second receptor was identified that preferentially bind with IGF-II (Rechler *et al.*, 1980). The subsequent purification of each of these

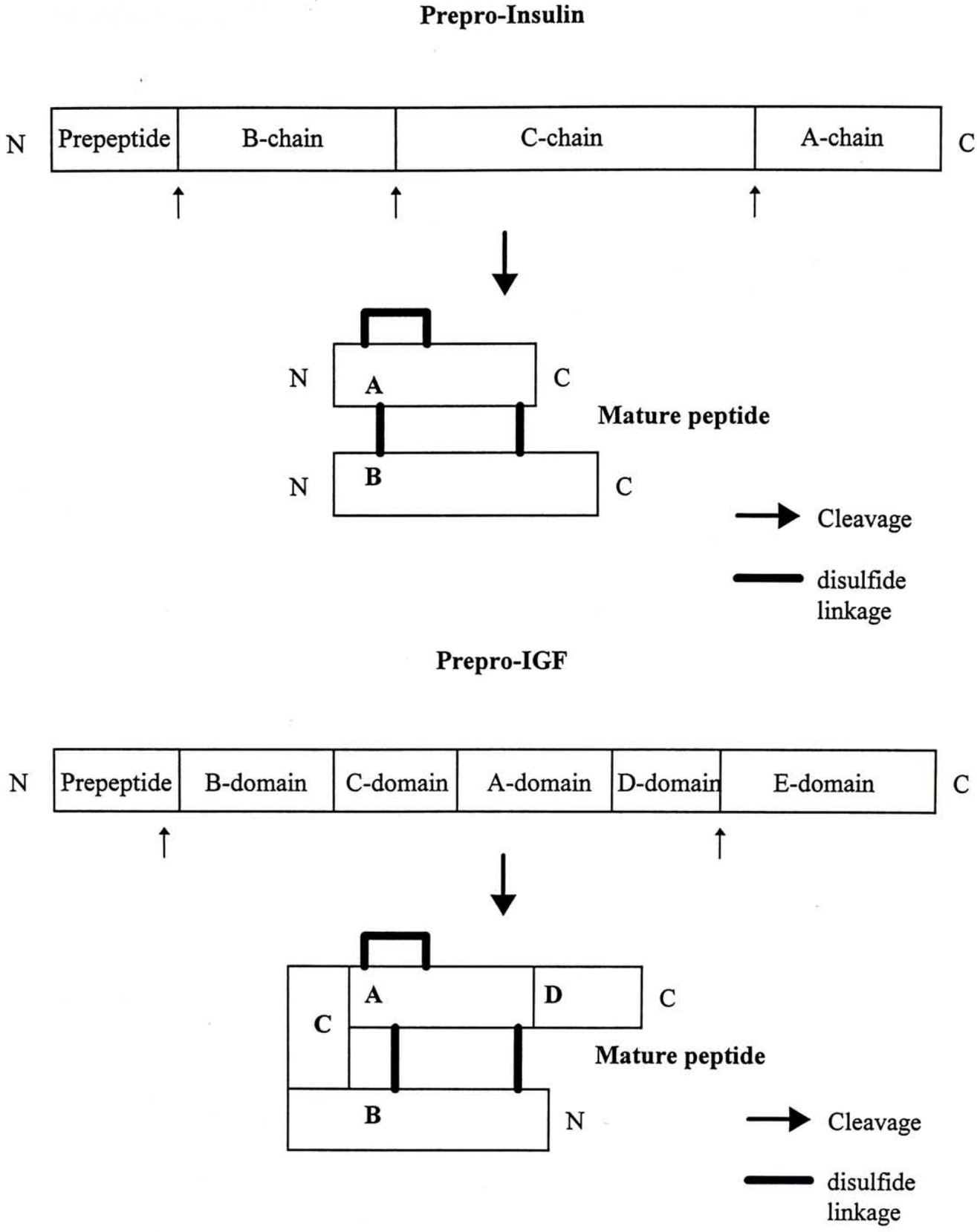

Fig. 1.1 Proteolytic processing of insulin and IGF. Approximate cleavage locations are indicated by arrows. The IGF and insulin regions are indicated and termed with historic nomenclature (chains and domains). The diagrams below each arrow depict the fates and relationships of the domains after cleavage. Inter- and intra-chain disulfide bonds are indicated by bold lines (Lowe, 1996).

receptors and cloning of their cDNAs demonstrated that each was the distinct product of a unique gene (Macdonald *et al.*, 1988; Morgan *et al.*, 1987; Ullrich *et al.*, 1986).

1.2.3 IGF Binding Proteins

Unlike insulin, circulating IGFs are bound to carrier proteins, now known as IGFBPs. The first indication of the existence of IGFBPs came in the mid 1970s, when it was shown that radioiodinated IGFs complexed with serum proteins, resulting in recovery after neutral chromatography of IGFs in the molecular massed in the range of 50-150 kDa but not 7.5 kDa (Zapf *et al.*, 1975). It was soon suggested that these serum carrier proteins had at least two functions: prolongation of the half-life of circulating IGFs and neutralization of their metabolic effects (Zapf *et al.*, 1979). Through protein purification and cloning experiments, it is now known that the IGFBP family comprises at least six members and a variety of functions have been ascribed to these proteins (Bach and Rechler, 1995; Baxter, 1993; Jones and Clemmons, 1995).

1.3 Origin and Production of IGFs

1.3.1 The Hypothalamo-Pituitary-GH-IGF-I Axis

The hypothalamus produces GH releasing hormone (GHRH) and somatostatin (SRIF).The hormones together modulate the secretion of GH so that it is released in pulses from the pituitary (Fig. 1.2). GHRH induces GH secretion while SHIR inhibits GH secretion. The pulsatility of GH release seems to be essential for normal growth (Rees and Maxwell, 1996).

Approximately half of the circulating GH is bound to a specific high affinity binding protein, GH binding protein (GHBP). GHBP contains amino acid sequences

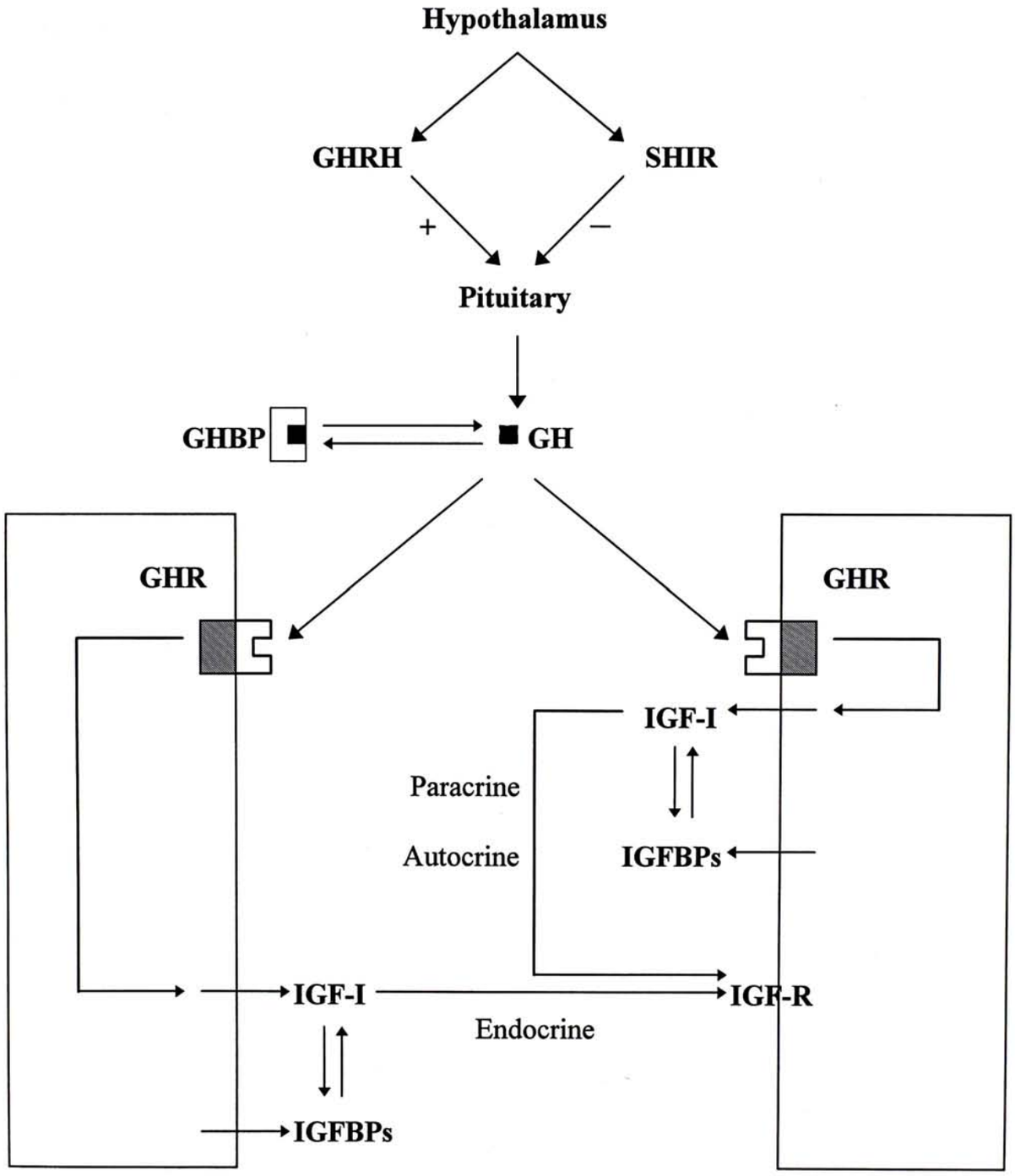

Fig. 1.2 Overview of the hypothalamo-pituitary-GH-IGF-I axis.

that are identical to the extracellular GH-binding domain of the GH receptor (GHR) and it is thought that GHBP arises by proteolytic cleavage from the GHR. The avidity and specificity of GHBP is similar to that of GHR itself. It is thought, therefore, that GHBP may reflect GHR activity (Rosenfeld *et al.*, 1994). Besides modulating the interactions of GH with tissue receptors, GHBP holds the pulse of GH in the circulation and in the extracellular space and decrease clearance (Rosenfeld *et al.*, 1994).

Upon the binding of GH to the GHR, IGF-I production is stimulated. Initially, no concentrated tissue source could be discovered, but it was subsequently recognized that the liver was the primary source of circulating IGF-I. IGF-I produced in the liver is transported to various target tissue by IGFBPs through the circulation. The peptide appeared to function, therefore, as a classic endocrine hormone, produced in one place (in this case, the liver) and carried in the circulation to distinct sites of action. However, IGFs are also expressed in a variety of tissues, including heart, lung, kidney and skeletal muscle, where they can act in a paracrine and autocrine fashion in response to GH (Lowe, 1996; Rees and Maxwell, 1996). The autocrine and paracrine action of IGFs are proposed to have a role in organ specific growth, while endocrine IGFs are proposed to participate in the integrated whole-body growth. Circulating IGF-I may also feed back at the pituitary and hypothalamus level to inhibit GH secretion.

1.3.2 Factors Regulating IGF Production

GH sufficiency is not the only factor responsible for regulating IGF-I production, for it depends on other factors such as an appropriate nutritional status. Uncontrolled diabetes is associated with decreased IGF-I production, and this

contributes to the growth abnormalities observed in children with poorly controlled diabetes (Lolaczynski and Caro, 1994). Chronic undernutrition leads to reduced serum IGF-I. Malnutrition and diabetes, like GH, affect IGF-I production in many tissues. Even though GH secretion is altered by malnutrition and diabetes, treatment with GH is not able to correct these defects. Decreased IGF-I production secondary to nutritional deficiencies appears to reflect alterations in GH sensitivity. Studies showed that nutrient availability regulates GH action at multiple levels: liver GHR regulation, GH postreceptor events, and IGF-I gene expression. Protein deprivation also accelerates IGF-I clearance, modifies IGF-I interaction with binding proteins, and attenuates expression of biological actions of IGF-I (Ketelslegers *et al.*, 1995; Thissen *et al.*, 1994).

IGF-I expression is also developmentally regulated. However, fetal development is GH independent, so the developmental regulation of IGF-I expression in fetal and neonatal animals is secondary to factors other than GH. Hepatic production and circulating levels of IGF-I decline with age, primarily because of decreased GH secretion (Humbel, 1990).

In addition to GH and nutrition, there are other tissue-specific factors that play a part in regulating IGF-I production. For example, gonadotropins and sex steroids regulate IGF-I expression in uterus, ovary, and testis (Mauras *et al.*, 1996). In the skeletal system, estrogen, and parathyroid hormone are able to regulate IGF-I expression. Regulation of IGF-I production in a given tissue is therefore complex and subject to both systemic and local factors.

IGF-II is also expressed in a wide variety of tissues during fetal development. Postnatally, however, IGF-II expression is extremely limited in rodents, which has hindered the development of model systems to examine its regulation. In humans,

unlike rodents, IGF-II production continues into adult life. One clear difference between IGF-I and IGF-II is that IGF-II production is independent of GH (Lowe, 1996). This has led to the rash and generalized conclusion that IGF-II represents the fetal growth factor, whereas IGF-I is the corresponding growth factor after birth.

1.3.3 Expression of IGFs in the Central Nervous System

It was found that IGFs in the circulation are not the major source of IGFs for the central nervous system (CNS). IGF-I, IGF-II, IGF-I receptor, and some IGFBPs are expressed in many regions of the CNS beginning *in utero* (D'Ercole *et al.*, 1996).

The expression pattern of the IGF system proteins during brain growth suggests highly regulated and developmentally timed IGF actions on specific neural cell populations. IGF-I expression is predominantly in neurons and, in many brain regions, peaks in fashion temporally coincident with periods in development when neuron progenitor proliferation and/or neuritic outgrowth occurs. For example, in the hippocampus, IGF-I expression is high in several proliferative areas in the first 2 weeks of postnatal life, but peaks later in the dentate gyrus, where neuronal proliferation is more prolonged. Purkinje cells are the major cells of IGF-I expression in the cerebellum. IGF-I expression in Purkinje cells commences at about the time of birth, declines by postnatal day (P) 20, and continues throughout adulthood (Aguado *et al.*, 1992; Torres-Aleman *et al.*, 1994).

In contrast, IGF-II expression in the brain and head occurs predominately in mesenchymal and neural crest derivatives during embryonic life (Stylianopoulou *et al.*, 1988). In adult rodent and human brain, the expression of IGF-II appears to be confined to the choroid plexus, meninges, and blood vessels (Hynes *et al.*, 1988; Lee

et al., 1993). IGF-II in these sites likely accounts for the abundance of IGF-II in the cerebrospinal fluid.

While expression of IGF-I receptors appears ubiquitous, that of IGFBPs is characterized by regional and developmental specificity, and occurs coordinately with peaks of IGF expression.

1.4 Actions of IGFs

The actions of IGFs are now commonly classified into three classes: metabolic, mitogenic, and differentiative. It should be noted that generally insulin, IGF-I, and IGF-II can substitute each other in promoting all these effects, but with differing potencies. Insulin is more potent in stimulating metabolic effects, whereas IGFs are more potent in stimulating growth-promoting effects (Baxter, 1988).

1.4.1 Insulin-Like Metabolic Effects

The metabolic activities (mainly anabolic) includes insulin-like actions such as simulation of glucose oxidation, glycogen synthesis and amino acid transport. In some cell types (notably adipocytes) these functions may be mediated by cross-reaction of the IGFs at insulin receptors, but in other cells, the type I IGF receptor appears to be the mediator (Froesch and Zapf, 1985).

Insulin-like activity can also be seen *in vivo*: injection of IGF-I into rats or humans elicits a hypoglycaemic response similar to that caused by insulin, and with about 6% of the potency of insulin (Guler *et al.*, 1987; Zapf *et al.*, 1985).

1.4.2 Mitogenic Effects

The mitogenic activity of the IGFs has been demonstrated in many cell types, with increase in DNA, RNA, and protein synthesis, as well as in cell proliferation, stimulated by both IGF-I and IGF-II (Baxter, 1986). In contrast to other cells, the proliferation of lymphocytes was inhibited by IGF-I. In BALB/c-3T3 fibroblasts, the IGFs have been shown to exert their mitogenic activity in synergism with other peptides such as platelet-derived growth factor or fibroblast growth factor, which must first render the cells "competent" before the IGFs can stimulate DNA synthesis (Pledger *et al.*, 1978; Stiles *et al.*, 1979 Leof, 1982 #43). Recent research also suggested that IGFs and fibroblast growth factors induced cell proliferation in cultured utricular epithelial cells (Zheng *et al.*, 1997).

Gross somatic growth effects can also be seen *in vivo*, with IGF infusion over several days into hypophysectomized rats increasing tibial length, thymidine incorporation into cartilage, and body weight (Zapf *et al.*, 1985). Similar results were obtained in normal rats (Schoenle *et al.*, 1982) and again in hypophysectomized rats, but this time comparing the effects of IGF-I with those of IGF-II (Schoenle *et al.*, 1985). In this regard, IGF-II was found to be less potent than IGF-I.

1.4.3 Effects on Differentiation

Evidence is accumulating that IGFs can also have distinct effects on the differentiation of cells. Rat IGF-II stimulates neurite formation in chick sensory neurons (Bothwell, 1983), while both IGF-I and IGF-II promote myoblast differentiation to myotubes (Florini *et al.*, 1991; Stewart *et al.*, 1996; Tollefsen *et al.*, 1989). In the thyroid gland, IGF-I synergises with thyroid stimulating hormone in stimulating cell proliferation (Tramontano *et al.*, 1986); comparable effects have also

been described in the adrenal cortex (Morera *et al.*, 1986). Thus, it appears that full expression of the activity of some pituitary hormones on their target endocrine tissues (adrenal and thyroid) may require the concomitant action of IGF-I (Baxter, 1988).

1.4.4 IGFs in Reproductive System

The effects of IGFs on the reproductive systems are well established and a variety of differentiation functions are influenced by IGFs (LeRoith *et al.*, 1995). In ovarian granulosa cell, which is primarily involved in the developing ovarian follicle and corpus luteum, and which itself synthesizes estrogen and progesterone. IGF-I acted synergistically with follicle-stimulating hormone (FSH) to induce receptors for luteinising hormone (LH), increase cyclic AMP production, enhanced progesterone production, and stimulated steroidogenesis (Adashi *et al.*, 1985; Adashi *et al.*, 1992).

High levels of IGF-I, IGFBPs, and IGF-I receptor were also found in the uterus. In this organ, estrogen had a marked effect on the expression of IGF-I (Murphy and Friesen, 1988). IGF-I also stimulated biologic functions in the testes. IGF-I increased LH binding to Leydig cells and LH-mediated cAMP accumulation. Androgen production is also increased by IGF-I (Morera *et al.*, 1987). In summary, IGF-I plays an important role in testicular function.

1.4.5 IGF Actions in the Central Nervous System

Increasing evidence strongly supports a role for IGF-I in CNS development. IGFs were shown to stimulate the proliferation of neuron progenitors and/or the survival of neurons and oligodendrocytes, and in some cultured neurons, to stimulate function (D'Ercole *et al.*, 1996). Cultured E10 murine neuroepithelial cells appeared to be dependent on IGF-I for proliferation and survival (Drago *et al.*, 1991). Both IGF-I

and -II stimulated the proliferation of aggregate cultures of mouse E15-16 neuron and glial precursors (Lenoir and Honegger, 1983). In immortalized hypothalamic neurons, IGF-I appears to enhance survival by protecting against the effects of oxidants (Sortino and Canonico, 1996). Late gestation rat astrocytes have been shown to express IGF-I and -II receptors and to synthesize DNA in response to IGFs (IGF-I>IGF-II) (Ballotti *et al.*, 1987). Evidence also suggests that IGFs stimulate the differentiation of certain neuronal populations. For example, IGFs stimulate hypertrophic growth of some neurons. IGF-I stimulates cell hypertrophy, neuritic outgrowth, and glutamic acid decarboxylase expression in cultured cortical neurons (Aizeman and De Vellis, 1987).

In vivo studies indicated that IGF-I can affect the development of most, if not all, brain regions, and suggested to us that the cerebral cortex and cerebellum are especially sensitive to IGF-I actions (Torres-Aleman *et al.*, 1994). *In vivo* growth-promoting actions of IGF-I is resulted result from its capacity to increase neuron number, at least in certain populations, and from its potent stimulation of myelination (D'Ercole *et al.*, 1996).

1.5 Transgenic and Knockout Animal Models for IGFs

To understand the mechanisms of IGF actions in multiple biological processes *in vivo*, trangenic (overexpression of IGFs gain of function) and knockout (targeted disruption of IGFs gene loss of function) animals, mainly mice, were studied.

As expected, transgenic mice overexpressing human IGF-I showed enhanced growth (Behringer *et al.*, 1990; D'Ercole, 1993; Mathews *et al.*, 1988), however trangenic mice with over produced IGF-II did not (Van Bull-Offers *et al.*, 1995; Ward *et al.*, 1994). A 1.3-fold increase in weight occurred in one transgenic line in which

serum IGF-I levels were 1.5 times above controls, although this was manifested as elective organomegaly rather than as an increase in skeletal size (Mathews *et al.*, 1988). In contrast, overexpression of human IGF-II led to little change in weight or length compared with nontransgenic controls, despite up to an eight-fold rise in circulating levels (Van Bull-Offers *et al.*, 1995; Ward *et al.*, 1994). Lack of a positive effect on growth was also seen in pituitary-ablated rats administered IGF-II (Glasscock *et al.*, 1992). These results, together with observations showing that genetic IGF-II deficiency in mice did not alter growth after birth (Dechiara *et al.*, 1990).

Knockout mice lacking IGF-I showed markedly diminished postnatal growth (Baker *et al.*, 1993; Powell-Braxton *et al.*, 1993), but also had impaired fetal growth and development (Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993). Depending on the study, mice heterozygous for a disrupted IGF-I gene were 80-100% the size of wild-type littermates at birth (Baker *et al.*, 1993; Powell-Braxton *et al.*, 1993), while homozygous mutant animals were ~60% of normal weight and length (Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993).

A decline in growth rate beginning at embryonic *day 13.5* was noted in homozygotes (Baker *et al.*, 1993). The IGF-I-deficient mice additionally showed diminished viability, with perinatal death being attributed to muscle hypoplasia and decreased maturation of the lungs (Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993).

The postulated function of IGF-II as a key fetal growth factor (Daughaday *et al.*, 1982; Moses *et al.*, 1980) was proven when a growth-deficiency phenotype was created in mice by targeted disruption of the IGF-II gene (Dechiara *et al.*, 1990). Mice lacking IGF-II were ~60% the size of wild-type littermates at birth (Dechiara *et al.*, 1990; Liu *et al.*, 1993), and diminished growth was apparent as early as embryonic

day 11. As noted above, a normal growth rate was observed after birth, and the animals were otherwise viable and fertile (Dechiara *et al.*, 1990).

Mice lacking both IGF-I and IGF-II, or animals deficient in both IGF-II and the IGF-I receptor, had an exacerbated dwarf phenotype and were ~30% of wild-type size at term (Liu *et al.*, 1993). These mice died at birth of lung failure and had many of the same anatomic defects as were found in IGF-I receptor mutants (Liu *et al.*, 1993). Taken together, these results demonstrate that expression of each IGF and each receptor is required for normal embryonic and fetal growth.

1.6 Molecular Biology of IGFs

IGF-I and -II are each the product of a single gene, localized on the long arm of chromosome 12 and on the short arm of chromosome 11, respectively in human. The IGF-II gene is contiguous by linked with the insulin gene and tyrosine hydroxylase gene, the order being 5'-tyrosine hydroxylase-insulin-IGF-II-3' (Humbel, 1990).

1.6.1 Structure of the IGF Genes

IGF-I Gene Structures

The genes encoding IGF-I were characterized in human (Rotwein *et al.*, 1986), rat (Shimatsu and Rotwein, 1987), and chicken (Kajimoto and Rotwein, 1991). The mammalian genes consist of six exons arranged as shown in Fig. 1.3. Exon 1 and 2 encode alternative 5'-untranslated regions (UTRs) and AUG translation initiation codons that are in-frame with the open reading frame contained in exon 3 and 4, and presumably encode divergent signal peptides. Exon 3 encodes the remainder of the signal peptide and part of the B domain of the mature peptide. Exon 4 encodes the

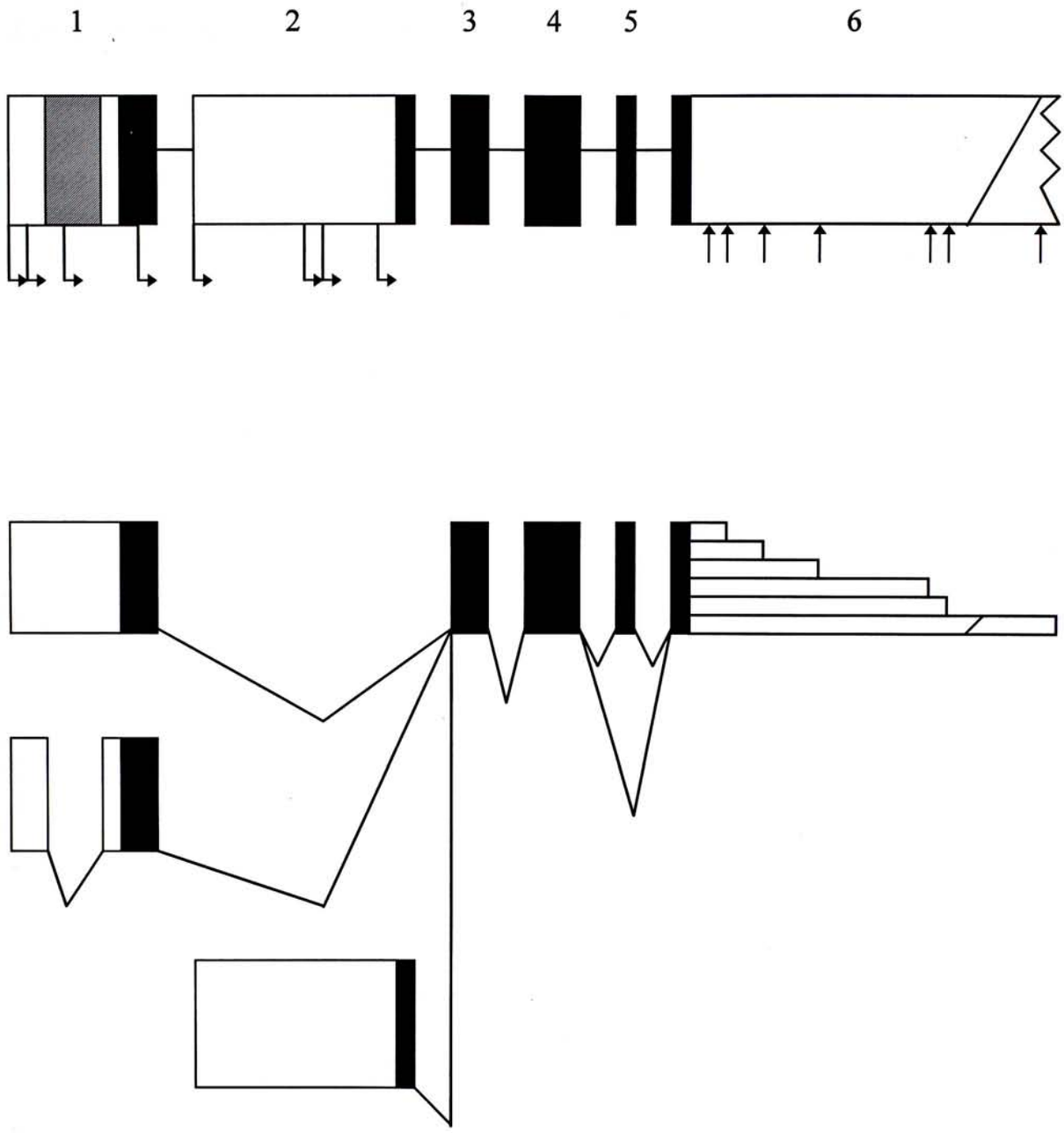

Fig. 1.3 Schematic organization of the rat IGF-I gene. Exons (boxes) are to scale; introns (lines) are not. Shaded areas represent portions of the prepro-IGF-I open reading frame. Horizontal arrows below exons 1 and 2 represent transcription start sites. Vertical arrows below exon 6 represent polyadenylation sites. Patterns of differential leader exon usage, splicing, and polyadenylation sites usage in different IGF-I mRNAs are diagrammed in the lower portion of the figure (Shimatsu and Rotwein, 1987).

remainder of the B domain, the C, A, and D domains, and the amino-terminus of the carboxy-terminal E domain. Exon 5 in the rat and mouse genes encodes a portion of E domain. In the human IGF-I gene, the splice junction at the position corresponding to the end of exon 5 in the rodent genes has apparently been mutated so that the human exon 5 is longer and encodes an extended E domain and the 3'-UTR sequences (Lowe *et al.*, 1988). In all IGF-I genes, exon 6 encodes the carboxy-terminus of the E domain and the 3'-UTR.

In terms of overall organization, the chicken IGF-I gene is similar to mammalian IGF-I genes, except for the apparent absence of exons encoding alternative 5'-UTR/prepeptide coding regions and E domain sequences (corresponding to exons 2 and 5) (Kajimoto and Rotwein, 1991). It is conceivable that those exons was emerged during mammalian evolution as a mechanism for the diversification of signal and E domain sequences to produce multiple forms of IGF-I prepro- and prohormones which may have a variety functions.

IGF-II Gene Structures

As illustrated by Fig. 1.4, IGF-II gene structure is as complicated as the IGF-I gene in terms of overall organization. Exons 1 through 6 encode divergent 5'UTR sequences. Exons 1, 4, 5, and 6 are leader exons flanked by independent transcription start sites and are, therefore, analogous to exons 1 and 2 of the mammalian IGF-I gene. Exons 2 and 3 are present in all exons 1 containing mRNAs. Exon 7 encodes the prepeptide and part of the B domain and is thus analagous to exon 3 of the IGF-I gene. Exon 8 encodes the remainder of the B domain, the C, A, and D domains, and the amino terminus of the IGF-II gene E domain, and is thus similar to exon 4 of the IGF-I gene. Exon 9 of the IGF-II gene, like exon 6 of the IGF-I gene,

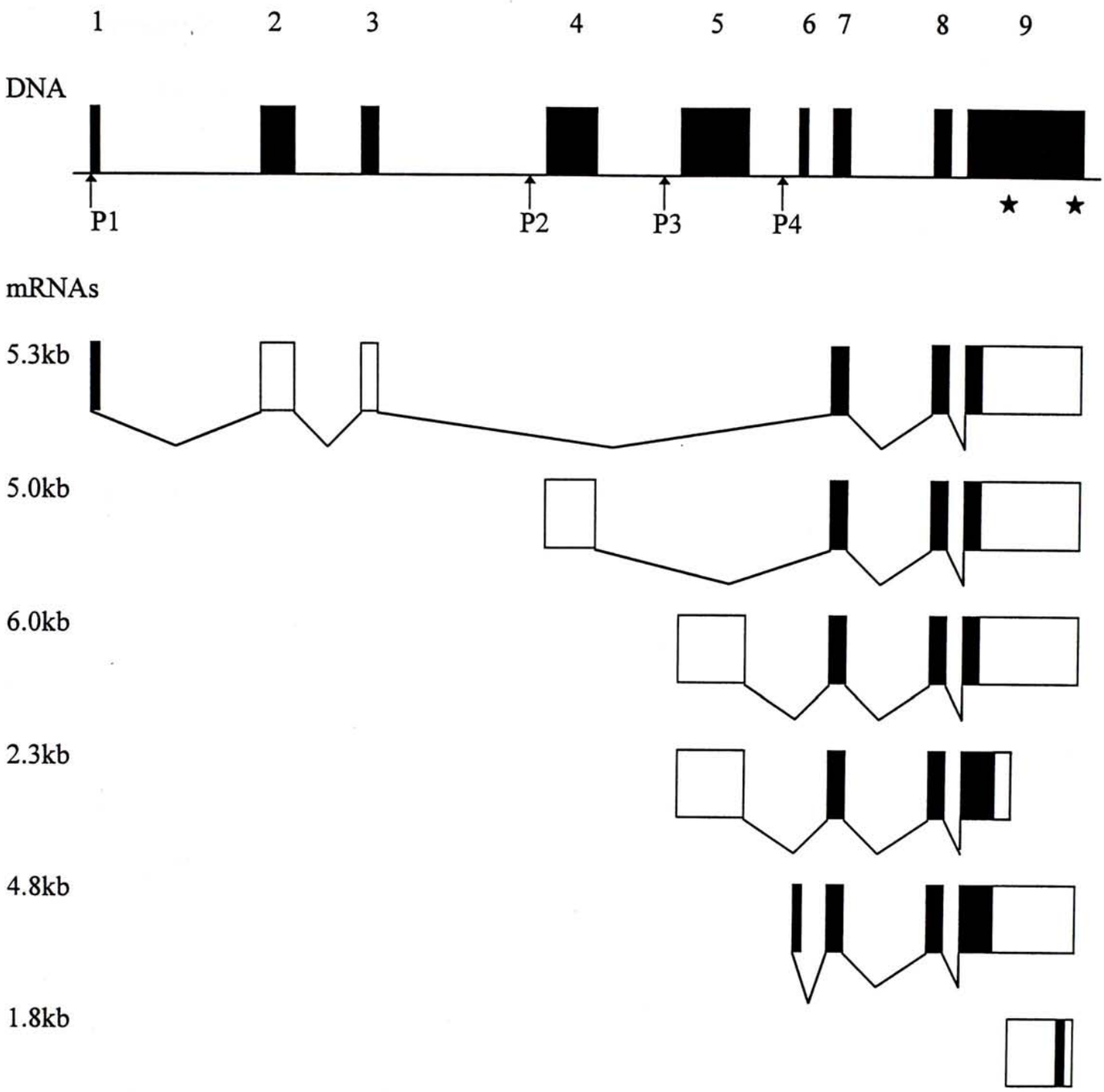

Fig. 1.4 Human IGF-II gene structure. Exons (boxes) and introns (lines) are to scale. Shaded areas represent portions of the preproIGF-II open reading frame. Vertical arrows correspond to promoters, and asterisks to polyadenylation sites. Patterns of differential leader exon usage, splicing, and polyadenylation site usage in different IGF-II mRNA species and their sizes are shown in the lower portion of the figure.

encodes the remainder of the E domain and the 3'UTR. Unlike the IGF-I gene, the IGF-II genes analyzed to date (human and rat) lack exons that encode alternate prepeptide or E domain sequence (Sussenbach, 1989). Exon 1, 2, and 3 are absent in the rat gene and are inactive in the mouse gene, occurring as "pseudoexons" (Rotwein and Hall, 1990).

1.6.2 Expression of IGF Genes

IGF-I Gene Expression

The presence of multiple leader and alternatively spliced exons in the IGF-I gene results in a complex pattern of expression illustrated in the lower portion of Fig. 1.3. In addition, multiple transcription initiation sites utilized in both leader exons of the human (Jansen *et al.*, 1991; Kim *et al.*, 1991), rat and mouse genes (Adamo *et al.*, 1991; Hall *et al.*, 1992). The majority of transcription in exon 1 is initiated at two sites which, in the rat gene, are separated by ~100 base pairs (bp). This pattern of transcription initiation may be due to the lack of core promoter elements such as TATA and CAATT motifs that generally occur ~30 and 80 bp, respectively, upstream of unique transcription start sites. Transcription in exon 2 is primarily initiated at a cluster of sites ~60 bp upstream of the 3' end of exon 2, presumably as a result of the presence of TATA and CAATT-like elements at appropriate locations upstream of the exons 2 transcription start sites. The combination of multiple transcription start sites in exons 1 and 2 and differential splicing in exon 1 produces a collection of IGF-I mRNAs which contain divergent 5'-UTRs. As a result, different IGF-I mRNAs are translated using different translation initiation codons, one in each exons 1, 2 and 3.

Alternative splicing of exon 5 has been demonstrated in human (Rotwein, 1986) and rat (Lowe *et al.*, 1988) IGF-I genes and is presumed to occur also in the

mouse and ovine genes. The exon 5 sequence, when present, alter the proIGF-I open reading frame and the sequence of the E domain. The E domain amino acid sequence encoded by the exon 5 containing mRNA lacks the one or two N-linked glycosylation sites found in the E domain sequence encoded by exon-5-lacking mRNAs. The two N-linked glycosylation sites in the pertinent rat E domain sequence were shown to be functional *in vitro* (Bach *et al.*, 1990).

As illustrated in Fig. 3, exon 6 contains multiple polyadenylation sites. IGF-I mRNAs which vary from less than 1 kilobase (kb) to more than 7 kb in length are produced through the use of these different processing sites (Lund *et al.*, 1989). The high-molecular-weight IGF-I species tends to predominate in extrahepatic tissues. The different lengths of 3'-UTR can affect the stability of IGF-I mRNA.

IGF-II Gene Expression

As shown in the lower portion of Fig. 1.4, transcription in exons 1, 4, 5 and 6 results in IGF-II m RNAs with different 5'UTRs and overall lengths of 6.0, 5.3, 5.0, and 4.8 kb. Exon 5 containing transcripts are polyadenylated at one of two sites in exon 9. Use of the more proximal site produces a 2.2-kb IGF-II m RNA. Interestingly, endonucleolytic cleavage of the exon 9 sequence results in a presumably nonfunctional 1.8-kb mRNA (Meinsma *et al.*, 1991). All of the IGF-II transcripts just described (with the exception of the exon 9 derived 1.8-kb species) encode the same IGF-II precursor, in contrast to the various IGF-I mRNA species. The use of an alternative splice acceptor site at the 5' end of exon 8, however, results in an IGF-II mRNA sequence that would encode an additional 4 amino acids in the B domain (Jansen *et al.*, 1985). The variant IGF-II peptide presumably encoded by this alternatively spliced mRNA has, in fact, been found in serum (Hampton *et al.*, 1989).

1.6.3 Regulation of IGF Gene Expression

The complex organization of these genes provides ample opportunities for control of gene expression at multiple levels. It is important to realize that regulation at one level can influence regulation at a different level. While such regulatory interactions are characteristic of both the IGF-I and IGF-II gene, they are particularly evident in the case of IGF-I gene expression. Firstly, the presence of distinct transcription initiation sites in the multiple leader exons of mammalian IGF-I genes and their differential expression under various conditions suggest that the IGF-I gene contain two promoters that regulate transcription initiation at the transcription start sites in exons 1 and 2. The choice of transcription start site influences the length and the sequence of the 5'-UTR (LeRoith *et al.*, 1995).

Secondly, the translatability of, and the proteins encoded by, IGF-I mRNAs with different 5'UTRs may itself be subjected to regulation. *In vivo* studies suggested that IGF-I mRNAs containing these different 5'UTR sequences may be differentially translated (Foyt *et al.*, 1992). The different prepeptides encoded by the various IGF-I mRNA species may be subjected to differential processing, with possible ramifications for IGF-I action (LeRoith and Roberts, 1991)

Finally, IGF-I biosynthesis might be controlled at the level of mRNA stability, as reflected in the apparent differential stability and tissue-specific expression of the high-molecular-weight IGF-I mRNA. Thus, one level of control of IGF-I gene expression may involve regulation of 3' end processing and poladenylation site selection.

Exon 1, 4, 5, and 6 of the IGF-II gene are flanked by distinct promoters, which have been designated P1 through 4. Transcripts initiated at the P2, P3, and P4 promoters predominate in most embryonic tissues. Hepatic transcripts, which are

primarily responsible for circulating IGF-II, are initiated at the P1 promoter after birth (dePagter-Holthuisen *et al.*, 1988). Both the P3 and P4 promoters contain TATA elements as well as binding sites for transcription factors such as SP1, whereas the P1 and P2 promoters lack TATA motifs and exhibit disperse transcription initiation (Matsuguchi *et al.*, 1990).

Some evidence were obtained to show IGF-II gene expression being regulated at the translational level. Specifically, mRNAs transcribed from the P2 and P4 promoters, which have shorter 5'UTRs, are enriched in the polysomal RNA fraction in comparsion to P3-derived transcripts, which contain longer 5'UTR sequences (Matsuguchi *et al.*, 1990). Another possible modulator of IGF-II mRNA translatability is the generation of the 1.8-kb fragment of the exon 9 sequence, in that removal of this 3'UTR sequence from the parental mRNA could affect the stability or translatability of the latter. Recent studies reported that the 1.8-kb sequence is itself not translated (Nielsen and Chistiansen, 1992).

1.7 IGF Receptors

The biological actions of IGFs are mediated by specific cell membrane receptors. The IGF-I receptor and IGF-II receptor can be distinguished by their primary structure, relative affinities for their respective ligands, as well as by immunological means (Rutanen and Pekonen, 1990).

1.7.1 IGF-I Receptor

The IGF-I receptor (Fig. 1.5), like most growth factor receptors, possess tyrosine kinase activity and is responsible for signaling the mitogenic and possibly all of the growth-promoting effects of IGF-I and IGF-II. It is a heterotetramer composed

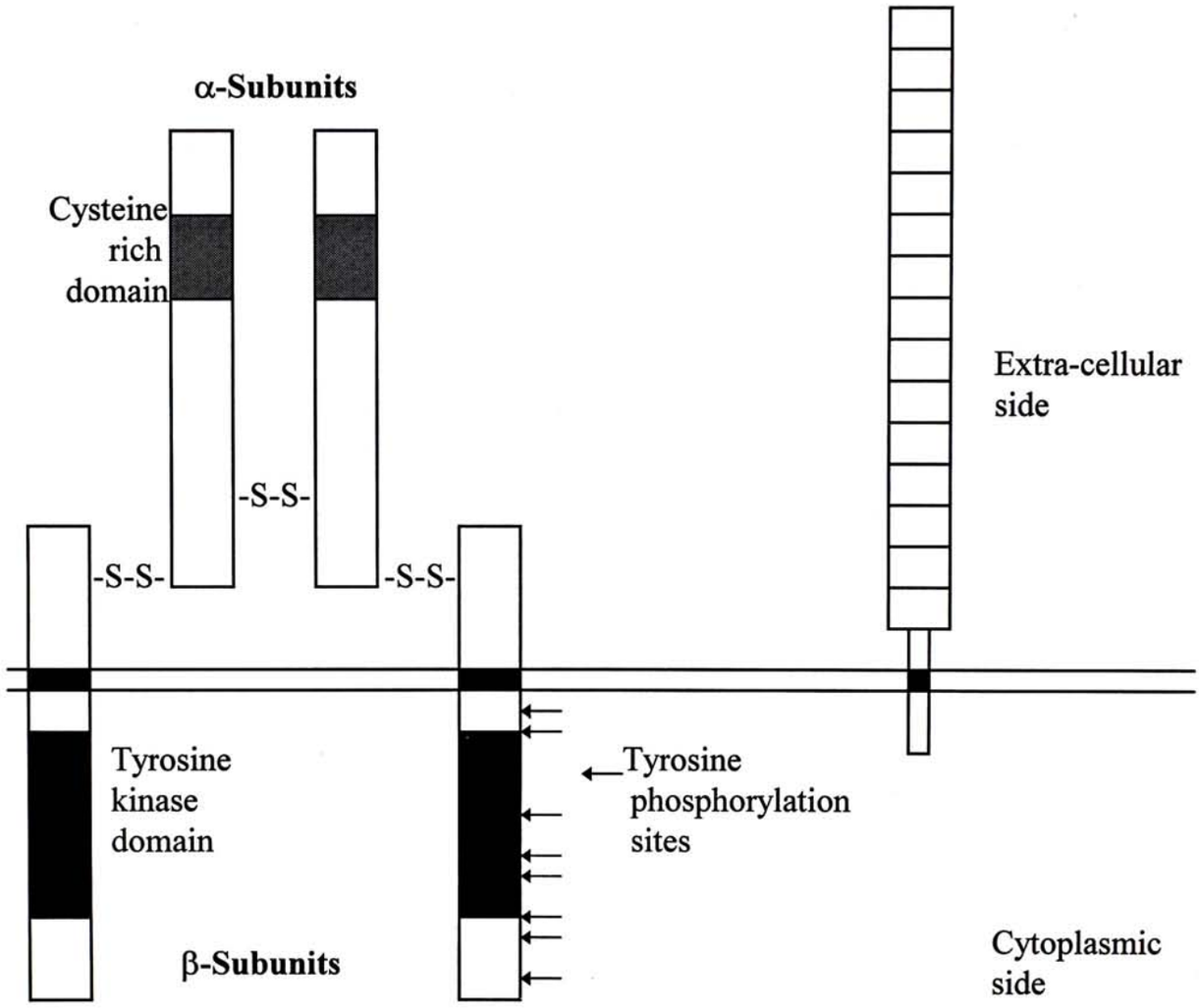

Fig. 1.5 IGF-I receptor *(Left)* and IGF-II/mannose-6-phosphate receptor *(Right)*. The insulin receptor also is a heterotetramer with a structure very similar to that of the IGF-I receptor (D'Ercole, 1996).

of paired, disulfide-linked α and β subunits. The α subunits span the cell membrane and include a tyrosine kinase and a carboxy terminus containing three tyrosine residues that are important in signaling. The entire receptor is the product of a single gene and shares structural homology with the insulin receptor (D'Ercole, 1996).

1.7.2 IGF-II Receptor

The IGF-II receptor (Fig. 1.5) is identical to mannose-6-phosphate receptor. It translocates proteins containing mannose-6-phosphate moieties and IGF-II to lysosomes for degradation. There is little evidence to a support a signaling function for this receptor, although such as function has not been excluded. Thus, IGF-II receptor do not seem to mediate the growth-promoting actions of IGFs (D'Ercole, 1996).

1.8 IGFBPs

Six distinct IGFBPs have been identified so far. They are numbered IGFBP-1 to 6 in the order in which their primary sequences were determined. One of the best studied is IGFBP-3 which appears to serve as an intravascular storage reservoir for IGFs. Serum levels of IGFBP-3 are responsive to GH. In states of GH depletion, IGF-I binds to IGFBP-3 with the sebsequent association of an acid-labile subunit to form a ternary complex (Lowe, 1996). About 75% of the IGF-I and IGF-II in the circulation is carried in these complexes. Free in plasma, the half-life of IGF-I is about 10 minutes, but in ternary complexes it is 12 to 15 hours. The ternary complex cannot cross the endothelial barrier and is thus confined to the circulation (Rutanen and Pekonen, 1990).

IGFBP-1, 2, and 4 are also present in the circulation. These binding proteins are capable of crossing the vascular endothelium, so IGFs bound to them instead of IGFBP-3 could be transported from the plasma to target cells. In case of IGFBP-2 and 4, subsequent proteolytic cleavage drastically reduces binding affinity, allowing IGFs to bind to the IGF receptors (Bach and Rechler, 1995).

A second role for IGFBPs is to both inhibit and augment IGF action. In some cases, depending on the experimental conditions, the same binding protein can either promote or inhibit IGF activity. When added in large concentrations to cell cultures, IGFBP-1, 2, 3 and 4 inhibit IGFs, presumably by binding to them and limiting their access to the IGF-I receptor, whereas IGFBP-1, 3, and 5 also promote IGF actions (also see Table 1.1) (Lowe, 1996).

Table 1.1 Characteristics of IGFBPs (Adapted from Lowe, 1996)

IGFBP-1	Found in high concentrations in amniotic fluid Secreted by hepatocytes, decidua Regulated by insulin, cAMP, and other factors
IGFBP-2	Found in serum, CSF, seminal plasma Secreted by many cell types Expression higher in fetal than in adult tissues
IGFBP-3	Major binding protein in serum Secreted by hepatocytes and other cells Serum levels are GH-dependent
IGFBP-4	mRNA present in many tissues, greatest abundance in liver Inhibits IGF action in many cell types
IGFBP-5	mRNA is most abundant in kidney but present in many tissues Serum levels are low Associates with the extracellular matrix
IGFBP-6	Present in serum and CSF mRNA is abundant in many tissues Significantly higher affnity for IGF-II than for IGF-I

1.9 Teleost IGFs

In the past, most of the studies on IGFs have been focused on mammals. Until recently, more research work has been focused on other non-mammalian species, notably fish.

1.9.1 The GH-IGF-Axis in Teleost

The basic mechanism of GH-IGF axis is also shown to be operative but in a different manner in teleost. In addition to IGF-I, IGF-II also show GH-dependence (Shamblott *et al.*, 1995). Both IGF-I and GH were found to be related to somatic growth (Gray and Kelly, 1991; McCormick *et al.*, 1992; Skyrud *et al.*, 1989).

RNase protection assay was used to study the effect of bovine GH on the levels of IGF-I and IGF-II mRNA in juvenile rainbow trout (*Oncorhynchus mykiss*) (Shamblott *et al.*, 1995). A single i.p. injection of bovine GH at a dose of 10ug/g body weight resulted in significant increase in IGF-I and IGF-II mRNA levels in liver when compared with control. In addition to IGF-I, the GH-dependent appearance of IGF-II mRNA in the liver suggests important roles for this peptide hormone exclusive of IGF-I.

Radioimmunoassay was used to study the effect of salmon GH on plasma level of immunoreactive IGF-I in rainbow trout (Moriyama, 1995). Following oral or i.p. administration of salmon GH at a dose of 0.1 or 1.0ug/g body weight, the plasma IGF-I levels were significantly elevated. The results indicate that plasma IGF-I in salmonid fish is under GH control and that oral administration of the hormone is followed by longer-lasting effects than those achieved by i.p. injection.

Coho salmon (*Oncorhynchus kisutch*) received implants of osmotic minipumps containing recombinant bovine IGF-I increased both linear growth and

growth rate in weight. However, high doses of IGF-I resulted in hypoglycemia and death (McCormick *et al.*, 1992). Also, GH and IGF-I act in a synergistic mode in stimulating sulfate uptake by branchial cartilage *in vitro* in common carp (*Cyprinus carpio*).

Like mammals, nutritional status is also an important factor in GH and IGF-I regulation in teleost (Duan and Hirano, 1992; Duan and Plisetskaya, 1993; Pérez-Sánchez *et al.*, 1995). In eel (*Anguilla japonica*), the hepatic sulfation activity was significantly decreased by hypophysectomy or fasting for 14 days. This experiment suggested that the sulfation activity was regulated by pituitary function and nutritional status (Duan and Hirano, 1992). In salmon, food deprivation primarily reduces IGF-I mRNA expression in the liver which results, most probably, in a decline in systemic IGF-I levels and consequently leads to the retarded growth of salmon (Duan and Plisetskaya, 1993).

All these results showed that the growth-promoting actions and the basic mechanism of GH-IGF axis in mammal are generally true in fish.

1.9.2 Osmoregulation and Other Biological Actions of IGF in Teleost

In 1993, Sakamoto and Hirano studied the importance of GH-IGF-I axis in osmoregulation in specific organs in fish. The IGF-I mRNA in liver, gill and kidney of rainbow trout were measured by Northern analysis. After GH injection, IGF-I mRNA increased signficantly in these tissues. Transferring trout from fresh water to 80% sea water showed increase of their circulating GH after 1 day. IGF-I mRNA was altered significantly in the liver but were found to be induced in gill and kidney after 1 day and 8 days respectively. These observations suggested that the IGF-I gene was expressed differently among these organs during sea water adaptation. Therefore, GH

may stimulate hypoosmoregulation by inducing local IGF-I expression in osmoregulatory organs such as kidney and gill (Sakamoto and Hirano, 1993).

IGFs are also involved in the reproductive physiology in teleost. In spermatogenesis of rainbow trout, IGFs stimulate DNA synthesis of male germ cells by interacting directly with these cells through IGF-I receptor (Loir and Le Gac, 1994). In red seabream (*Pagrus major*), IGF-I and -II induced final maturation of oocytes *in vitro* (Kagawa *et al.*, 1994).

It was also shown that IGFs play a role in regulating production of new neurons in the teleost retina (Mack *et al.*, 1995) and the role of IGF-I in the regulation of brain growth, development and, possibly, some neurotrophic and neuromodulatory mechanism (Leibush *et al.*, 1996).

1.9.3 Molecular Biology of IGFs in Teleost

The presence of both IGF-I and IGF-II in teleost suggested that the divergence of IGFs occurred early in vertebrate evolution (Duguay *et al.*, 1996; Shamblott and Chen, 1992).

A portion of the IGF-I cDNA (including the B, C, and A domains, ~120bp) was amlified by RT-PCR from juvenile rainbow trout and was used as a probe to isolate trout IGF cDNAs. After cloning and DNA sequencing, 4 different forms of IGF-I mRNA (with different lengths in the E domain) and 1 form of IGF-II mRNA were identified (Shamblott and Chen, 1993). In the past, it was believed that IGF-II did not exist in fish, IGF-I and II were diverged only after mammalian radiation. This was the first time an IGF-II mRNA was identified in teleost (Shamblott and Chen, 1992).

Until now, only two nonalleic IGF-I genes (IGF-I.1 and IGF-I.2) were isolated from chum salmon (*Oncorhynchus tshawytscha*) (Kavsan *et al.*, 1994; Kavsan *et al.*, 1993). The IGF-I.2 gene differ from IGF-I.1 gene in the E peptide coding portion, in that it lacks one codon present in the IGF-I.1 gene and contains two potential splice donor sites at the 3' end of exon 3 rather than one site in IGF-I.1. It was speculated that the expression of these two IGF-I genes could give rise to six different forms of IGF-I mRNA in chum salmon.

For non-salmonids, common carp was found to express only one form of IGF-I cDNA (Liang *et al.*, 1996). RT-PCR showed that the liver of both sexes is the major site of IGF-I. expression

1.9.4 IGFBPs and IGF Receptors in Teleost

Western blot analysis using ^{125}I-labeled human IGF-I identified at least three major forms of IGFBPs in the plasma of 4 different teleost species (coho salmon (*Oncorhynchus kisutch*), striped bass (*Morone saxatilis),* tilapia (*Oreochromis mossambicus)* and longjawed mudsucker (*Gillichthys mirabilis*) . One form was about 40-50kDa, possibly GH dependent and seemed to be the teleost version of mammalian IGFBP-3. The second and third forms had sizes of 29 kDa and 31 kDa. They might be the teleost version of mammalian IGFBP-1 and -2 (Kelly *et al.*, 1992).

In another independent study using Western blot analysis, the binding of ^{125}I-labeled human IGF-I to trout serum gave positive bands of variable size. The 41.5 kDa and 38.5 kDa bands might correspond to IGF binding protein 3, whereas the 34 kDa, 30 kDa, and 24 kDa bands might correspond to IGF binding protein- 2, -1 and -4 (Niu and Le Bail, 1993).

Competitive binding studies and affinity labeling of brain membranes from 3 primitive vertebrates sea scorpion (*Cottus scorpius)*, ray (*Raja clavata*) and Atlantic hagfish (*Myxine glutinosa)* identified an IGF-I receptor by the size of its α subunit and its binding specificity to both IGF-I and IGF-II peptides (Darkenberg *et al.*, 1993).

1.10 Rationale and Aim of the Present Study

In the past, it was generally believed that IGF-II evolved after the divergence of mammals from other chorates (Shamblott and Chen, 1992). Until recently, IGFs have been identified in a limited number of nonmammalian species. Multiple forms of IGF-I and one form of IGF-II cDNA sequence were identified in rainbow trout (Shamblott and Chen, 1993). Two IGF-I genes were cloned in the genome of chum salmon (Kavsan *et al.*, 1994; Kavsan *et al.*, 1993).

In common carp, however, only one subtype of IGF-I and no IGF-II was found so far (Liang *et al.*, 1996). On the other hand, genomic Southern analysis performed on common carp demonstrated that there could be more than one IGF gene in the genome of common carp (unpublished work). The solution of this apparent dichotomy is dependent upon the isolation and cloning of IGF gene(s) from common carp. The aim of this study is to screen a common carp genomic library to see if we could identify the IGF gene(s) in the genome of common carp. Genomic Southern analysis was also used to detect the IGF-II gene using a rtIGF-II cDNA probe.

The IGF-I promoter region of fish exhibit many similarities revealed that the regulation of IGF-I expression is conserved among the teleost. For example, the promoter region of IGF-I gene in several fish species have been cloned and characterized (Funkenstein *et al.*, 1996; Kavsan *et al.*, 1993; Koval *et al.*, 1994; Kulik *et al.*, 1995). To see whether the promoter is also conserved in common carp and

study the promoter activity of the common carp IGF-I gene, we must first clone the promoter of common carp. In this study, we use PCR to amplify the upstream 5'-flanking region of the common carp genomic DNA.

In fish, the basic mechanism of GH-IGF axis is shown to be operative but in a different manner. In addition to IGF-I, IGF-II also show GH-dependence (Shamblott *et al.*, 1995). It was suggested that both IGF-I and IGF-II may have growth effects in teleost (Duguay *et al.*, 1996; Shamblott and Chen, 1992) Both IGF-I and GH were found to be related to somatic growth (Gray and Kelly, 1991; McCormick *et al.*, 1992; Skyrud *et al.*, 1989). To prove the GH-IGF-I axis work also in the common carp, IGF-I gene expression study was carried out using Northern blot analysis and RT-PCR.

CHAPTER 2 SEARCH OF IGF-I PROMOTER BY GENOMIC DNA POLYMERASE CHAIN REACTION

2.1 Introduction

The 5' regions of the human and rat IGF-I genes exhibit many similarities. Both genes contain two leader exons (1 and 2) that encode different 5'-UTR sequences (Rotwein *et al.*, 1986; Shimatsu and Rotwein, 1987) and that contain multiple transcription start sites (Jansen *et al.*, 1991). All transcript contain upstream, in frame translation initiation codons and therfore may encode different signal peptides. The nucleotide sequences of human exons 1 and 2 are highly homologous to the corresponding regions of the rat IGF-I gene. No concensus TATA box or AT-rich regions are present within the first 50 nucleotides upstream of any of the exon 1 transcription start sites determined, nor are these regions particularly GC-rich, suggesting that the exon 1 promoter regions belong to the class of TATA-less non-GC-rich promoters. Potential TATA- and CCAAT-like elements are present upstream of the major exon 2 start site in rat (Adamo *et al.*, 1991), but the role of these in exon 2 transcription has yet to determined.

Several IGF-I promoter regions were cloned and characterized in fish species (Kavsan *et al.*, 1993; Koval *et al.*, 1994; Kulik *et al.*, 1995; Funkenstein *et al.*, 1996). The sequences were found to be similar among the teleosts and indicated that the regulation of expression is identical in fish. In common carp, an IGF-I Ea2 cDNA was isolated (Liang *et al.*, 1996). To study and see whether the IGF-I promoter region of common carp is similar to other fish species, we cloned the 5'-flanking region by PCR according to the known sequence of the common carp IGF-I Ea2 cDNA.

2.2 Materials

DNA grinding buffer (Section 2.3.1)
0.1M Tris
0.05M Na_2EDTA
0.2M NaCl
1% SDS
10mg/ml Proteinase K (Boehringer Mannheim)

10M Ammonium acetate (Section 2.3.1)
770g ammonium acetate
Add ddH_2O to 1 liter, sterilize by filtration.

50X Tris acetate (TAE) (Section 2.3.4)
242g Tris base
57.1 ml glacial acetic acid
100 ml 0.5M EDTA, pH 8.0
Add ddH_2O to 1 liter.

6X Loading buffer for agarose gel electrophoresis (Section 2.3.4)
0.25% bromophenol blue
0.25% xylene cyanol FF
40% (w/v) sucrose in water

Gel Solubilizer (Section 2.3.5)
6M NaI
50mM Tris-Cl, pH 8.0
0.05% Na_2SO_3
10 mM EDTA

Wash Buffer (Section 2.3.5)
20mM Tris-Cl, pH 8.0
1 mM EDTA
0.1mM NaCl
50% ethanol

10X Ligation Buffer (section 2.3.6)
60 mM Tris-HCl, pH 7.5
60 mM $MgCl_2$
50 mM NaCl
1 mg/ml bovine serum albumin
70 mM β-mercaptoethanol
1 mM ATP
20 mM dithiothreitol
10 mM spermidine

LB (Luria-Bertani) medium (Section 2.3.7)

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g

Add ddH_2O to 1 liter, sterilize the buffer by autoclaving for 20 min at 15lb/sq. in. liquid cycle.

LB plate (Section 2.3.7)

Composition the same as LB medium except with 1.5% agar.

Isopropylthio-β-D-galactoside (IPTG) (Section 2.3.7)

2g IPTG
Add H_2O to 10ml
Sterilize the solution by filtration through a 0.22 μm filter.

Xgal (5-bromo-4-chloro-3-indolyl-β-D galactoside) (Section 2.3.7)

200 mg 5-bromo-4-chloro-3-indolyl-β-D galactoside (Xgal)
Dissolve in 10ml dimethylformamide and store in a dark bottle.

Solution I (Section 2.3.8)

50mM Tris-Cl, pH 7.5
10mM EDTA
100μg/ml RNase A
Sterilize the buffer by autoclaving for 20 min at 15lb/sq. in. liquid cycle.

Solution II (Section 2.3.8)

0.2 N NaOH
1% SDS

Solution III (Section 2.3.8)

3M potassium acetate, pH 4.8

A mix-short (Section 2.3.11)

840 μM each dCTP, dGTP and dTTP
93.5 μM dATP
14μM ddATP
40 mM Tris-Cl, pH 7.6
50 mM NaCl

T mix-short (Section 2.3.11)

840 μM each dCTP, dGTP and dATP
93.5 μM dTTP
14μM ddTTP
40 mM Tris-Cl, pH 7.6
50 mM NaCl

G mix-short (Section 2.3.11)
840 μM each dCTP, dATP and dTTP
93.5 μM dGTP
14μM ddGTP
40 mM Tris-Cl, pH 7.6
50 mM NaCl

C mix-short (Section 2.3.11)
840 μM each dATP, dGTP and dTTP
93.5 μM dCTP
17μM ddCTP
40 mM Tris-Cl, pH 7.6
50 mM NaCl

Enzyme dilution buffer (Section 2.3.11)
20mM Tris-Cl, pH 7.5
5 mM dithiothreitol (DTT)
100μg bovine serum albumin/ml
5% glycerol

Annealing Buffer (Section 2.3.11)
1 M Tris-Cl, pH 7.6
100mM $MgCl_2$
160mM DTT

Labeling Mix-dATP (Section 2.3.11)
1.375μM each dCTP,dGTP and dTTP
333.5 mM NaCl

Stop solution (Section 2.3.11)
0.3 % bromophenol blue
0.3% xylene cyanol FF
10 mM EDTA, pH 7.5
97.5% deionized formamide

5X Tris-borate (TBE) (Section 2.3.11)
54g Tris base
27.5g boric acid
20ml 0.5M EDTA, pH 8.0
Add ddH_2O to 1 liter.

20% Acrylalmide for sequencing gel (Section 2.3.11)

acrylamide	96.5g
methylene-bis-acrylamide	3.35g
ultra-pure urea	233.5g
5X TBE	100ml
H_2O	to 500ml

Urea mix for sequencing gel (Section 2.3.11)

urea	233.5g
5X TBE	100ml
H_2O	to 500ml

2.3 Methods

2.3.1 Preparation of Genomic DNA from Carp Testis

Genomic DNA of common carp was prepared according to the method described by Scott *et al.* (1985). In brief, testis tissue (0.5 g) was dropped into a mortar which has been filled with liquid nitrogen. The tissue was ground to powder and then 40 ml of grinding buffer (Section 2.2) was added slowly. The mixture was ground until homogeneous and then poured into a 150 ml Ehrlenmeyer flask. It was then incubated at 65°C with shaking for 3 hr. Four mg Proteinase K (Boehringer Mannheim) was further added and the mixture was incubated at 65°C with shaking overnight. The mixture was cooled and transferred to a centrifuge tube. An equal volume of phenol equilibrated with 0.5 M Tris-Cl (pH 8.0) was added and the tube was placed on a roller apparatus for 1 hr. The aqueous and organic phase was separated by centrifugation at 5,000g for 15 min. The aqueous phase was transferred to a clean tube using wide bore pipette tip and the phenol extraction was repeated twice. After phenol extraction, the aqueous phase was transferred to a fresh centrifuge tube. Ammonium acetate (10 M, 0.2 volume) (Section 2.2) and 2 volume of ethanol was added and mixed thoroughly. The DNA thread formed immediately was removed by a heat-bended Pasteur pipette. The DNA was washed by 70% ethanol and then collected by centrifugation. The DNA pellet was then dissolved in an appropriate amount of TE (pH 8.0). The absorbance of the DNA at 260 nm and 280nm was measured. The ratio of A_{260} to A_{280} should be greater than 1.75. A lower ratio is an indication that significant amounts of protein remain in the preparation.

2.3.2 Restriction Enzyme Digestion of Genomic DNA

Genomic DNA samples were digested by restriction enzymes. About 1 μg of genomic DNA was digested with excess amount of restriction enzymes. *Eco* RI, *Hin*d III and *Xba* I were used to digest samples of the genomic DNA in 50 μl of the corresponding buffer. The reaction mixtures were incubated at 37°C for 4 hr. The digested DNA samples were precipitated and dissolved in 10 μl sterilized water.

2.3.3 Polymerase Chain Reaction

2.3.3.1 Ligation of the Cassette to Digested Genomic DNA

Five μl of the digested DNA samples were ligated to the 50 ng Cassette with restriction site corresponding to *Eco* RI, *Hin*d III and *Xba* I in 15 μl of recommended ligation solution provided by the PCR *in vitro* Cloning Kit (Takara, Japan). The reaction mixture were incubated at 16°C for 30 min. The ligated DNA samples were precipitated and dissolved in 5 μl sterilized water.

2.3.3.2 Amplification by PCR

One μl of the ligated DNA samples were used for the first PCR and 1μl of the first PCR reaction mixture was used for the second PCR, using a PTC-100 thermal cycler (MJ Research, USA): 94 °C, 1 min; 55 °C, 2 min; 72 °C, 3 min; for 25 cycles. In each PCR reaction, 2.5 units of *Takara Taq* polymerase were used and 20 pmole of each primer were used in a reaction of 100 μl. IGF-I specific primers (S1 and S2) were designed from the coding region of common carp cDNA signal peptide (Liang *et al.*, 1996). The position of the primers are shown in Fig. 2.1. The Cassette Primers were obtained from the kit (Takara, Japan).

Fig. 2.1 Nucleotide sequence and the predicted amino acid sequence of the common carp IGF-I cDNA clone (Liang *et al.*, 1996). Labeled arrows above the sequence represent the positions and 5' to 3' direction of the primers used in Takara PCR and DNA sequencing.

The nucleotide sequences of primer sets for PCR are:

First round of PCR,

IGF-I specific primer (S1): 5'CCCCTGGAAGAAATGTCCGCTAGA3', and

Cassette Primer (C1): 5'GTACATATTGTCGTTAGAACGCG3'.

Second round of PCR,

IGF-I specific primer (S2): 5'AGACATCCCTTGGGTCTCCAGCAA3', and

Cassette Primer (C1): 5'TAATACGACTCACTATAGGGAGA3'.

2.3.4 Agarose Gel Electrophoresis

Ten µl of the final PCR reaction mixture and 6X gel-loading buffer (Section 2.2) was added to a final concentration of 1X. The gel was prepared with 1.2% (w/v) agarose dissolved in 1X TAE buffer (Section 2.2). Electrophoresis was performed at a constant voltage of 100V in a gel tank with 1X TAE. After electrophoresis, the gel was stained in ethidium bromide (EtBr) solution (10mg/ml) and visualized on a Fotodyne UV transilluminator at 300nm.

2.3.5 Gene Clean Using Sephaglas™ BandPrep Kit (Pharmacia)

The agarose gel containing the desired DNA band was excised, weighed and put into a 1.5 ml microfuge tube where 250 µl of Gel Solubilizer (Section 2.2) was added, and the mixture was incubated at 60°C for 5-10 min until the agarose slice dissolved. Five µl of Sephaglas BP was added to bind the DNA, the tube was vortexed gently and incubated for 5 min at room temperature. The tube was centrifuged for 10 sec at full speed in a microcentrifuge and the supernatant was carefully removed. The Sephaglas pellet was then washed with 40 µl of Wash Buffer (Section 2.2). The tube

was centrifuged at full speed for 10 sec and the supernatant was carefully removed. This washing step was repeated twice. The Sephaglas pellet was allowed to air dry for 10 min to remove any residual ethanol. Finally, the DNA was eluted in 20 µl preheated water (55-65°C).

2.3.6 Cloning of PCR Products

Fifteen µl of the PCR product (from Section 2.3.4) isolated was added to 50 ng of the pCR® 2.1 vector and mixed with 4 Weiss units of T4 DNA Ligase was used in 20 µl of the recommended ligation buffer (Section 2.2) provided by the Original TA Cloning® Kit (Invitrogen). The ligation mixture was incubated overnight at 14°C.

2.3.7 Transformation of Competent Cell (Heat Shock Method)

DH5α competent cells were used the for transformation with the plasmid vector. The competent cells which were stored in -70°C was thawed at room temperature until the cell suspension is just thawed. Then the cells were placed on ice. Twenty µl of the ligation mixture (Section 2.3.6) were added to 100µl competent cells and mixed by swirling. The tube was placed on ice for 30-60 min. The cells were heat shocked in 42°C for 90 sec and then quick chilled on ice for a few min. Nine hundred µl of LB medium (Section 2.2) was added to the cells and incubated at 37°C with moderate agitation for 60 min to allow the bacteria to recover and to express the antibiotic resistance gene encoded by the plasmid. One hundred µl of the transformed competent cells with 20 µl of 100mM IPTG (Section 2.2) and 50 µl of 2%(w/v) X-gal (Section 2.2) were spreaded onto a LB agar plate (Section 2.2) containing Ampicillin

(0.1 mg/ml). The plate was then incubated at 37°C overnight (Sambrook *et al.*, 1989). The white colonies were picked for plasmid minipreparation.

2.3.8 Small Scale Alkali Preparation of Plasmid DNA

To confirm that the colonies (from Section 2.3.7) obtained were positives, the plasmid DNA of these colonies were prepared by alkaline lysis (Sambrook *et al.*, 1989). A single colony of bacteria was transferred into 2ml of LB medium in snap-capped 15 ml culture tube. The culture was incubated at 37°C overnight with vigorous (250 rpm) shaking. One and a half ml was transferred to a microfuge tube and centrifuged at 12,000 g for 30 sec at 4°C. The remainder of the culture was stored at 4°C. The supernatant was discarded and the pellet resuspended in 100 µl of ice-cold Solution I (Section 2.2) by vigorous vortexing. Two hundred ml of freshly prepared Solution II (Section 2.2) were added and mixed by inverting the tubes several times. One hundred and fifty µl of ice-cold Solution III (Section 2.2) were then added and mixed by inverting the tube several times. The tube was kept on ice for 5 min and then centrifuged at 12,000g for 5min at 4°C, the supernatant was then transferred to a fresh tube. The plasmid DNA was precipitated by addition of 2 volumes of ethanol (95%) at room temperature for 10 min. It was then centrifuged at 12,000g for 5 min at 4°C. The supernatant was discarded and the pellet washed by 1 ml 70 % ethanol at 4°C. The ethanol was removed and the pellet was allowed to air dry for about 10 min under vacuum. Finally, the DNA pellet was dissolved in 50 µl ddH_2O and stored at -20°C.

2.3.9 Restriction Enzyme Digestion to Release the Insert

The sizes of the insert from positive clones (from Section 2.3.8) were checked after restriction enzyme digestion. Five μl of plasmid DNA were digested by 10 units of *Eco* RI (cloning site) in 20 μl corresponding buffer. The reaction mixture was incubated at 37°C for 4 hr. Agarose gel electrophoresis was performed using 10 μl of the digested plasmid on a 1.2% (w/v) agarose gel (Section 2.3.4).

2.3.10 Large Scale Plasmid Preparation of the Positive Clone Using

Large scale plasmid preparation was carried using the Wizard Maxiprep Kit (Promega). One hundred to five hundred ml overnight culture of *E. coli* were prepared and the bacteria was pelleted by centrifugation at 14,000g for 10 min at 4°C. The bacterial pellet was then completely resuspended in 15 ml of Cell Resuspension Solution (Solution I, Section 2.2). Fifteen ml of Cell Lysis Solution (Solution II, Section 2.2) were added and mixed gently, but thoroughly, by stirring or inverting until the solution becomes clear and viscous. Fifteen ml of Neutralization Solution (Solution III, Section 2.2) were added and mixed by inverting the centrifuge bottle several times. The mixture was filtered through filter paper (Whatman #1, GFA) in to a centrifuge bottle. Isopropanol (0.6 volume) was added and mixed by inversion. The mixture was stored at -20°C for at least 1 hr and then centrifuged at 14,000g for 15 min at 4°C to collect the plasmid. The supernatant was discarded and the DNA pellet was resuspended in 2 ml TE buffer. Ten ml of Wizard Maxipreps DNA Purification Resin were added to the DNA solution and mixed by swirling. For each maxiprep, one Wizard Maxicolumn was used. The Maxicolumn tip was inserted into the vacuum source. A vacuum was applied to pull the Resin/DNA mix into the Maxicolumn. The

Resin/DNA mix was washed by 12.5 ml Column Wash solution (Section 2.2) for two times and a vacuum was applied to draw the Wash solution through the Maxicolumn. To rinse the Resin, 5ml of 80 % ethanol were added to the Maxicolumn and a vacuum was applied to draw the ethanol through the Maxicolumn. The Resin was further by centrifugation at 2,500 rpm for 5 min.

One and a half ml preheated (65-70°C) ddH_2O (1.5 ml) were added to the Maxicolumn. After 1 min, the DNA was eluted by centrifuging the Maxicolumn/Reservoir at 2,500 rpm for 5 min. The plasmid DNA may then be stored at 4°C or -20°C.

2.3.11 DNA Sequencing of the Positive Clone Using the T7 DNA Polymerase Sequencing Kit (Pharmacia)

The concentration of the template was adjusted so that 32µl of ddH_2O contained 1.5 to 2 µg DNA. The double-stranded template was denatured by mixing 32µl clone and 8µl 2M NaOH in a microcentrifuge tube. The tube was then incubated at room temperature for 10 min. Seven µl of 3M sodium acetate (pH 4.8) and 4µl of distilled water was added and mixed. One hundred and twenty µl of 100% ethanol were added to the mixture. The content was mixed by inverting the tube several times, and placed on dry ice for 15 min. The DNA was then centrifuged down at full speed for 15 min. The supernatant was discarded and the pellet was washed with ice-cold 70% ethanol followed by recentrifugation for 10 min at full speed. The supernatant was discarded. The DNA pellet was dried under vacuum, and redissolved in 10 µl of ddH_2O.

The concentration of primer was adjusted to 2.5-5 μM. The primer and the Annealing Buffer (Section 2.2) were added to the resuspended template, as indicated below:

Template DNA	10μl
Primer	2μl
Annealing Buffer	2μl
Total volume	14μl

The tube was vortexed gently, then centrifuged briefly and incubated at 65°C for 5 min. The tube was transferred to a 37°C water bath and incubated for 10 min. The tube was then stored at room temperature for at least 5 min, and then centrifuged briefly. Two and a half μl respectively of A mix-short, C mix-short, G mix-short and T mix-short (Section 2.2) were transferred into separate microcentrifuge tubes.

To the tube containing the annealed template and primer, 3μl labeling mix (Section 2.2), 1μ labeled dNTP and 2μl of diluted T7 DNA Polymerase (4 units) were added. The components were mixed by gentle pipetting, and incubated at room temperature for 5 min. While this incubation was in progress, the four sequencing mixes were warmed by placing the microcentrifuge tubes at 37°C for at least 1 min.

After the 5-min incubation of the labeling reaction, 4.5 μl of this reaction were transferred into each of the four pre-warmed sequencing mixes and incubated at 37°C for 5 min. Five μl of Stop Solution (Section 2.2) were then added to each tube to terminate the labeling reaction. The tubes were heated at 75-80 °C for 2 min and 1.5 to 2μl of each heated sample were loaded into the appropriate well of a sequencing gel. To make a 8% sequencing gel (Sambrook *et al.*, 1989), 20ml 20 % acrylamide (Section 2.2), 30ml urea mix (Section 2.2) and 0.4ml 10% ammonium persulfate were

mixed. Fifty μl of TEMED were added to the mixture, mixed and immediately poured into the sequencing gel mold. Electrophoresis was performed at constant power of 55W with 1X TBE buffer.

The nucleotide sequences of primers for sequencing are:

Universal primer:	5'GTTTTCCCAGTCACGAC3',
Reverse primer:	5'CAGGAAACAGCTATGAC3'
pCR® 2.1 vector specific primer:	5'GCCAGTGTGCTGGAATT3'
IGF-I specific primer (S3):	5'AGATGCGGGCAATGTCACATCTCA5'.

The position of the IGF-I specific primer (S3) is shown in Fig. 2.1.

2.4 Results and Discussion

Fig. 2.2 shows the strategy of cloning the common carp IGF-I 5'-flanking region The common carp genomic DNA was first completely digested by selected restriction enzymes separately. Then, the cassette with the restriction site of corresponding restriction enzyme was ligated to the genomic DNA. Since the 5'end of the cassette is not phosphorylated, a nick will occur within the ligation site of the 5'-end of the cassette and 3'-end of the target DNA. In the first PCR, second strand synthesis from the cassette primer C1 was stopped at the nick so that non-specific amplification was prevented. Only the second strand synthesized from IGF-I gene specific primer S1 was utilized as the template of DNA synthesis of complementary strand from the cassette primer C1. Then, the unknown region was amplified in more specific and in higher yield during second PCR using inside primers (C2 and S2). Using common carp genomic DNA digested by *Hin*d III restriction enzyme as template, the PCR product was cloned into pCR® 2.1 vector. Fig. 2.3 shows the PCR products after agarose gel electrophoresis and EtBr staining. It was found that the one using *Hin*d III cut genomic DNA as template a the largest size PCR product of ~800 bp. Therefore, the corresponding band was excised and purified from the gel. The DNA fragment was then cloned into pCR® 2.1 vector and labeled as IGF1P.

Nucleotide sequence determination was carried out for this clone and the sequence is shown in Fig. 2.4. It was found that the insert includes 840 bp nucleotide sequence encoding the proximal 5'-UTR and the first part of the signal peptide of common carp IGF-I cDNA. Therefore, the clone, IGF1P, contains the 5'-flanking of the common carp IGF-I gene.

Sequence analysis (Fig. 2.5) revealed that the 5'-flanking region of common carp IGF-I gene is highly conserved when compared with the sequence of IGF-I gene

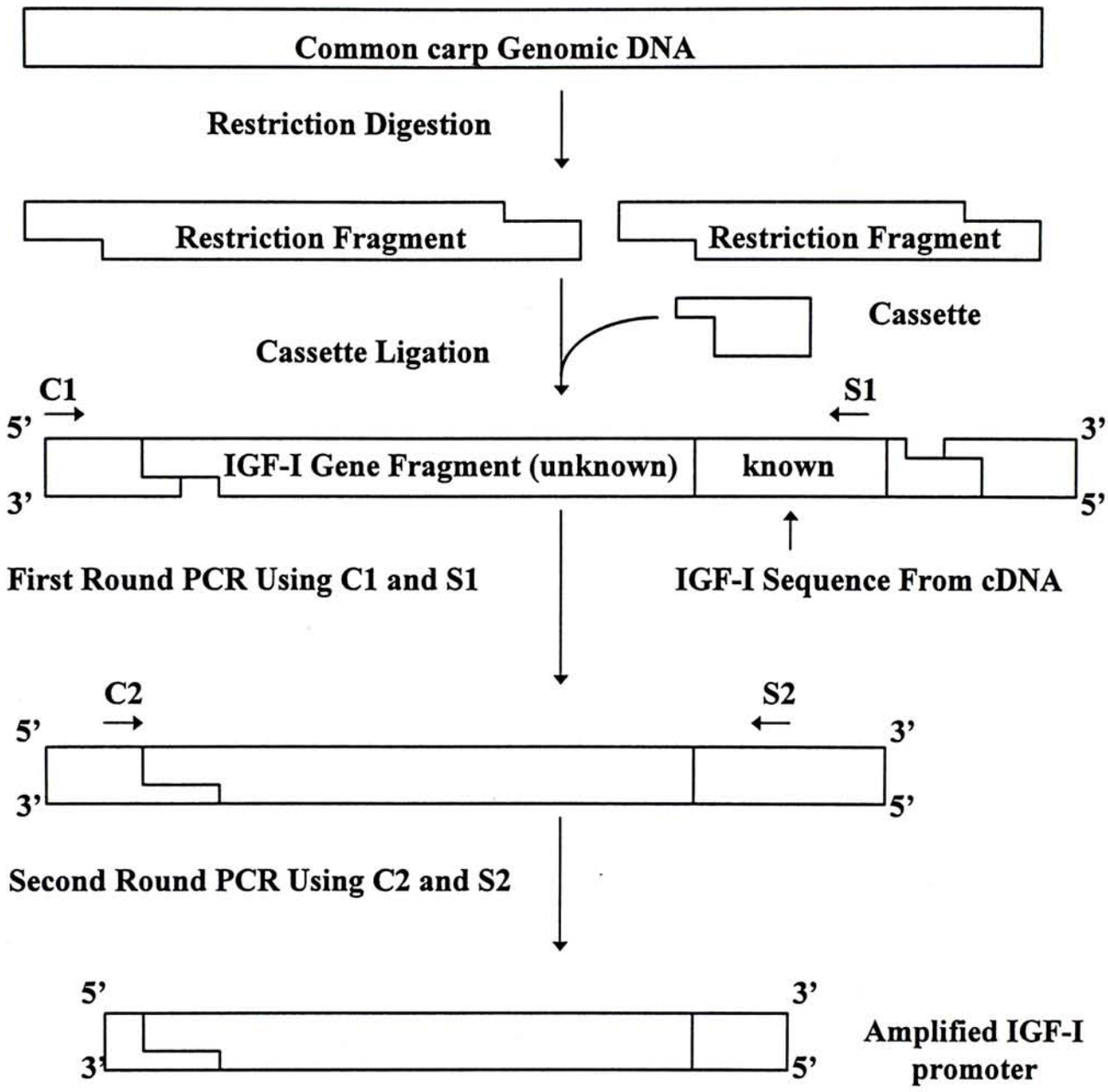

Fig. 2.2 Strategy of cloning the common carp IGF-I 5'-flanking region. Labeled arrows above the fragments represent the relative positions and 5' to 3' direction of the primers used in Takara PCR

Lane 1-- λ *Hind* III marker
Lane 2-- PCR product using *Eco* RI digested common carp genomic DNA as template
Lane 3-- PCR product using *Hind* III digested common carp genomic DNA as template (cloned & sequenced)
Lane 4-- PCR product using *Xba* I digested common carp genomic DNA as template
Lane 5-- 1 kb marker

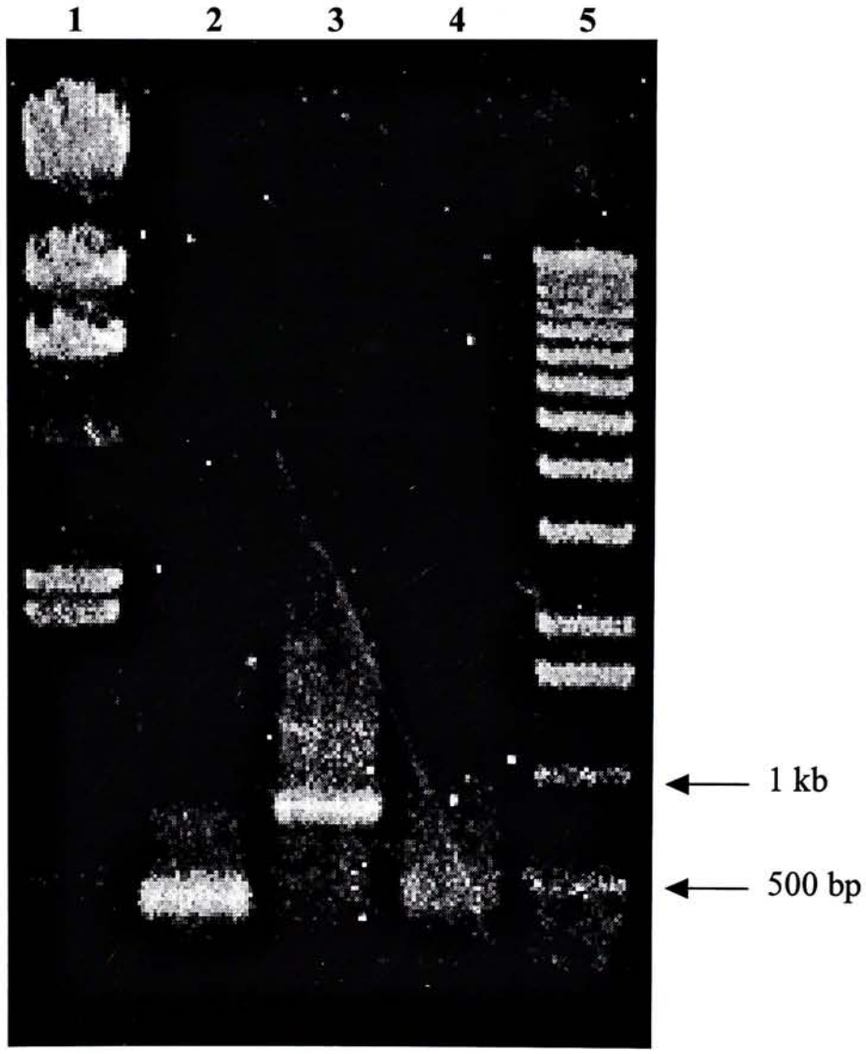

Fig. 2.3 The DNA gel (after EtBr staining) showing the 2nd round PCR products using gene specific primers (S1 and S2) designed from the common carp IGF-I cDNA clone and the cassette primers (C1 and C2) provided by the Takara kit.

840 AGCTTTTGAA GAGACGCGCG AAAAGAAGTC GCTTTGGTCT GAAGTGGAAA CCATACGATA

780 AACATCGAGG ATTCAAACGG GACATTGACG AGACAAATGC TGTCATTATT GAGCATTTTG

720 AATTATATTA GAGATATACT AGACATGGCT GCCACTGCTC AGTAACACAA ACCACTATCA

660 TTACAATTTT ATAGATATAT TACAAGAACT TTCAGTTTTA AAATCTTTTA GATGATGCTT

600 AGCTTTATCT CAGCTAATTG CGCCCCGAAA ACATAGGCTA CTATAGAAAT TCATTTAAAA

540 AAAGTTAATC TCATATAAAT GCATTAAAAA AGGCACACGC TGGCAAGTAT ACAGAGATGA

480 AAAAGACACC GTGCAAATTA CGCATGGGAG TGTCTCTAAA TTCAACTTCT GTCCTTTTCT

420 AAAAACTCAC CACTTTCTCT AACTCCCTTA TTCGTTTTCA TTCTGCAATG CAATCTTTTC

360 TGTTCTGTTA GTAAGTTCCA AAATGATCCA AATTCGAGCC TATTCTAAGA AACAGATGAC

300 GTTATTTGAC AGGGTGCCCA AAATCCTTAA TGAGTAACTT AGCAAGAGGA GAAGGCAAAT

240 GCTGCCCCAG CTGTTTCCTG TTGAAAATGT CTCTGTAATG TAGATAAATG TGAGGGATTT

180 TCTCTCCAAA TCCGTCTCCT GTTCGCTAAA TCTCACTTCT CCACAACGAG CCTGCGCAAT

Primer S3

120 GGAACAAAGT CGGAATATTG AGATGTGACA TTGCCCGCAT CTCATCCTCT TTCTTGCTTT

Primer S2

60 TTAATGACTT CAAACAAGTT CATTTTTGCT GGGCTTTTGC TGGAGACCCA AGGGATGTCT

MetThrS erAsnLysPh eIlePheAla GlyLeuLeuL euGluThrGl nGlyMetSer

Fig. 2.4 Nucleotide sequence of the 5'-flanking region of common carp IGF-I gene (clone IGF1P). Labeled arrows above the sequence represent the position and 5' to 3' direction of the primers used in Takara PCR and DNA sequencing. The ATG encoding the first Met in the IGF-I signal peptide is underlined; the nucleotide sequence of the signal peptide is boxed.

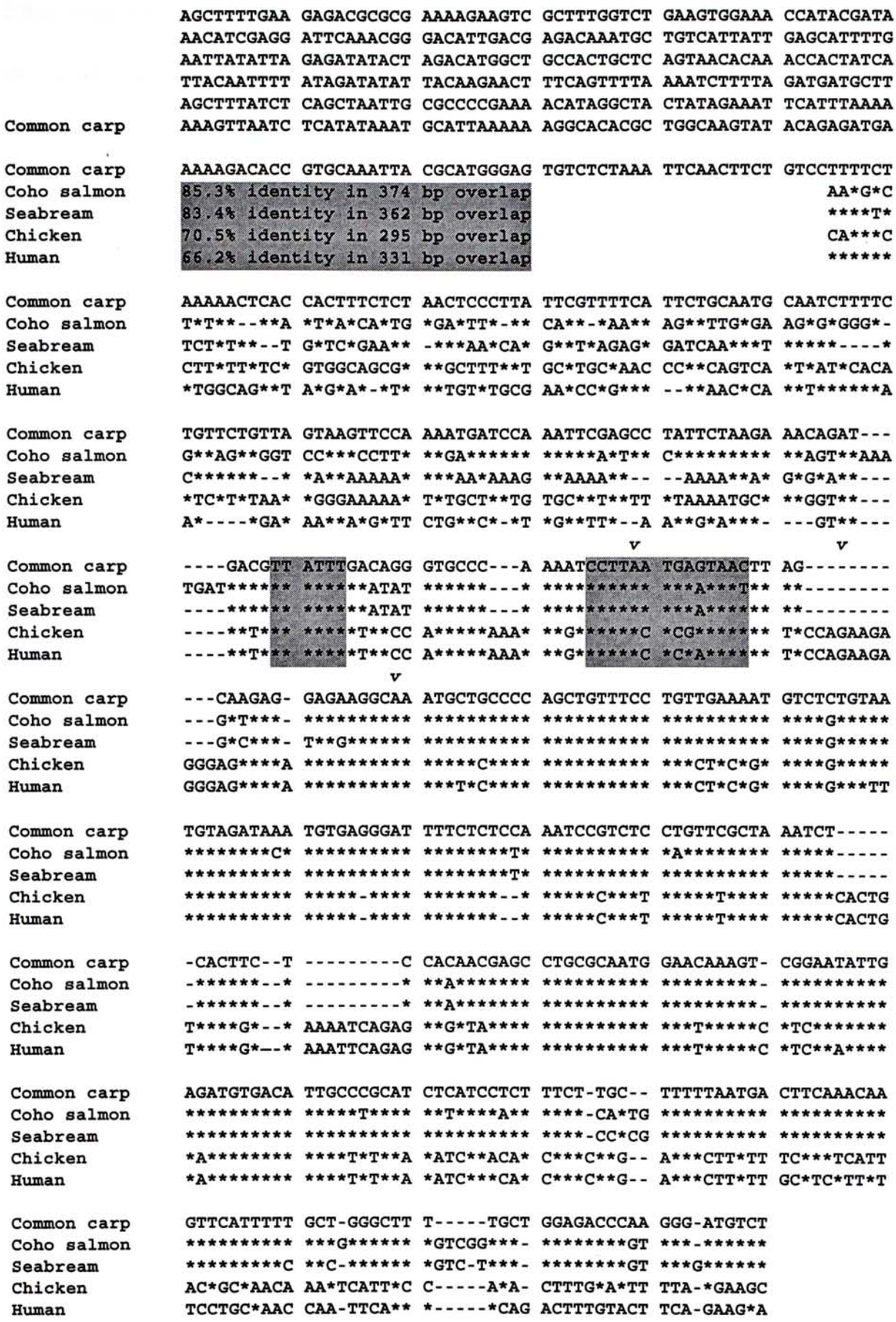

Fig. 2.5 Comparison of nucleotide sequences of the 5' flanking region in common carp IGF-I gene with similar regions of the coho salmon, seabream, chicken and human IGF-I genes; the corresponding percentage identity is shown. Identical nucleotides are indicated by *. Gaps are introduced to maximize homology and are indicated by -. Transcription start sites described Kajimoto and Rotwein (1991) for chicken IGF-I gene are marked by *v*. The TATA-like motif and HNF-1 sequences (Kajimoto and Rotwein, 1991) are shaded.

from coho salmon(Koval *et al.*, 1994), seabream (Funkenstein *et al.*, 1996), chicken (Kajimoto and Rotwein, 1991), and human (Rotwein *et al.*, 1986). Comparison of the region upstream from the first methionine with IGF-I sequence from the four vertebrates demonstrated a very highly conserved region up to the sequence TTTTCT, which start at -368 nucleotides upstream of the first methionine of the prepeptide. By contrast, the nucleotides upstream of the -368 nucleotides shows very little similarity among the four vertebrates. Within the conserved region, the common carp IGF-I 5'-flanking region has about 70 % identity with chicken and human and, even higher with more than 80 % identity among different fish species.

As in the coho salmon, seabream, chicken and human IGF-I genes (Kavsan *et al.*, 1993), there is a TATA-like consensus sequence approximately 250 bp 5' upstream of the first methionine of the common carp IGF-I gene. A putative initiator sequence, suggested for the coho salmon, seabream, chicken and human is also present in the common carp. This sequence, which is identical to that found in the chum salmon IGF-I gene (Kavsan *et al.*, 1994), contains a potential hepatocyte nuclear factor 1 (HNF-1) binding site (Kulik *et al.*, 1995). It was found that the HNF-1 has a potent stimulatory action the activity of the promoter of the salmon IGF-I gene in transient transfection experiments. Mutating this sequence to a variant which is no longer capable of exhibiting HNF-1 binding resulting in the loss of the stimulatory effect.

In conclusion, we have successfully cloned the promoter region of common carp IGF-I gene and it was used as a probe to characterize the IGF genomic clones obtained from screening of a common carp genomic library.

CHAPTER 3 ISOLATION OF GENOMIC CLONES CARRYING THE IGF-I GENE

3.1 Introduction

To examine the phylogeny, biochemistry and molecular biology of insulin-like growth factor (IGF) in local fish species, we started our studies using a local tropical fish, the common carp (*Cyprinus carpio*). We have demonstrated that IGF-I Ea2 form is the predominantly expressed form of IGF in common carp (Liang *et al.*, 1996). Multiple forms of IGF-I and one form of IGF-II cDNA sequences were identified in rainbow trout (Shamblott and Chen, 1993). Two nonallelic IGF-I genes were cloned in chum salmon (Kavsan *et al.*, 1994; Kavsan *et al.*, 1993). These two IGF-I genes appeared to be more compact than the mammalian and avain genes, being less than 20 kb in length. As in other species, however, the mature IGF-I peptide consists of 70 amino acids encoded by exon 2 and 3.

In common carp, however, only one subtype of IGF-I and no IGF-II was found so far. On the other hand, genomic Southern blot analysis performed on carp demonstrated that there could be more than one IGF gene in the genome of common carp. The solution of this apparent dicotomy is dependent upon the isolation and cloning of IGF gene(s) from common carp. In this study, we screened a common carp genomic library for IGF genes to see if we could identify the IGF gene or genes in the genome of common carp. The probe encodes conserved region of common carp IGF-I cDNA. Presumably, it can hybridize to both IGF-I and -II nucleotide sequences.

3.2 Materials

LB (Luria-Bertani) medium (Section 3.3.1)

Bacto-tryptone	10g
Bacto-yeast extract	5g
NaCl	10g

Add ddH_2O to 1 liter, sterilize the buffer by autoclaving for 20 min at 15lb/sq. in. liquid cycle.

LB plate (Section 3.3.1)

Composition the same as LB medium except with 1.5% agar.

NZY medium (Section 3.3.2 and 3.3.3)

NZ amine	10g
NaCl	5g
Bacto-yeast extract	5g
$MgSO_4.7H_2O$	2g

Add ddH_2O to 1 liter, sterilize the buffer by autoclaving for 20 min at 15lb/sq. in. liquid cycle.

NZY top agarose (Section 3.3.2 and 3.3.3)

Composition the same as NZY medium except with 0.7 % agarose.

NZY plate (Section 3.3.2 and 3.3.3)

Composition the same as NZY medium except with 1.5% agar.

Denaturation buffer (Section 3.3.3)

1.5M NaCl
0.5M NaOH

Neutralization buffer (Section 3.3.3)

1.5M NaCl
1M Tris-Cl, pH 7.5

20X SSC (Section 3.3.3)

175.3g NaCl
88.2g sodium citrate
Add ddH_2O to 1 liter, adjust pH to 7.0 with 10N NaOH, sterilize the buffer by autoclaving for 20 min at 15lb/sq. in. liquid cycle.

10% SDS (Section 3.3.3)

100g SDS
Add ddH_2O to 1 liter, heat to 68°C, adjust pH to 7.2.

SM (Section 3.3.2, 3.3.5 and 3.3.6)

NaCl	5.8g
$MgSO_4.7H_2O$	2g
1M Tris-Cl, pH7.5	50 ml
2% gelatin solution	5ml
ddH_2O to 1 liter	

Add H_2O to 1 liter, sterilize the buffer by autoclaving for 20 min at 15lb/sq. in. liquid cycle.

Binding Buffer (Section 3.3.6)
Solution of sodium perchlorate buffered with Tris-HCl.

Denaturing solution for alkaline (Section 3.3.8)
1.5 M NaCl
0.5M NaOH

Alkaline transfer buffer (Section 3.3.8)
0.25M NaOH
1.5 M NaCl

3.3 Methods

3.3.1 Preparation of the Plating Host Cells

Fifty ml LB medium (Section 3.2) (containing 0.2% maltose and 10 mM $MgSO_4$) in a sterile Erlenmeyer flask were inoculated with a single colony of the XL-1 blue bacterial host. The culture (50ml) was grown overnight with shaking at 30°C. This temperature ensured that the cells would not overgrow as phage can adhere to dead as well as live cells. The cells were centrifuged down in a sterile conical tube for 10 min at 2,000 rpm on the following day. The media were carefully removed and the cell pellet was gently resuspend in ~15 ml of 10 mM $MgSO_4$. The cells were then diluted to OD_{600}=0.5 with 10 mM $MgSO_4$ and stored at 4°C.

3.3.2 Phage Titering

Serial dilutions (10^{-1},10^{-2},10^{-3},10^{-4},10^{-5},10^{-6} and 10^{-7}) of an aliquot of the phage library or phage stocks were prepared in SM buffer (Section 3.2). Five µl of each dilution of phage were added to 100 µl OD_{600}=0.5 host cell in 7 separate culture tubes. After incubated at 37°C for 15 min , 3ml 48°C NZY top agarose (Section 3.2) was added and the content was plated on each 100 mm NZY agar plate (Section 3.2). The plates were allowed to harden at room temperature for 10min. The plates were then incubated at 37°C overnight. The number of plaques formed were counted and the plaque forming unit (p.f.u.) were determined.

3.3.3 Primary Screening of Common Carp Genomic Library

A common carp genomic library was obtained from Stratagene, La Jolla, USA. Lambda FIX II was used as vector in this library and the averaged insert size is about 15 kb. The library was plated out at a concentration of 50,000 p.f.u. per agar plate (150mm in diameter) with 600 µl OD_{600}=0.5 host cell/plate and 7 ml NZY top agar/plate. Totally 600,000 p.f.u. were spreaded on 12 NZY plates. The plates were incubated at 37°C until the plaques were just visible to the naked eye. The plates were then put in a refrigerator at 4°C. This step was important in preventing top agarose from sticking to the nylon filter. Dry nylon filter were laid onto the plates for 2 min and the filters were pricked with a needle through the membrane into the agar for orientation. Filters were peeled off from the plates and laid, plaque side up, on a 3MM paper saturated with denaturation solution (Section 3.2) for 2 min. The filters were then transferred to a 3MM paper saturated with neutralization buffer (Section 3.2). After 4 min, the filters were submerged in 2X SSC (Section 3.2) for 30 sec to rinse. The filters were then placed on dry 3 MM paper and air dried for about 25 min. The DNA was crosslinked to the filters for 4 min in an UV crosslinker (Fotodyne). The plates were stored in a 4 °C refrigerator.

The common carp IGF-I cDNA *Rsa* I fragment was first purified using the method described in Section 2.3.5 after agarose gel electrophoresis and then labeled with α^{32}P-dCTP using an Amersham nick translation kit (Section 3.3.4). The filters were prehybridized at 65°C for 1 hr and then hybridized at 65°C for 2hr in Rapid-hyb buffer (Amersham). After hybridization, the filters were washed twice in 2X SSC, 0.1% SDS (Section 3.2) at 65°C for 30 min and exposed to Kodak BIOMAX™ MR film at -70°C for 4 days between intensifying screens.

3.3.4 Preparation of Radioactive Nucleic Acid Probes

The radioactive nucleic acid probes were prepared using the Nick Translation Kit (N5000) of Amersham.

The DNA probes were diluted to a concentration of 50ng/μl in ddH_2O. In a 50 μl reaction, 5 μl of 1 μg DNA sample, 10 μl nucleotide buffer solution, 5 μl α-^{32}P dCTP (PB10205, Amersham), 25 μl water and 5 μl enzyme solution were added to a clean microcentrifuge tube. The content was mixed gently by pipetting up and down and then incubated at 15°C for 60 min. However, if desired, any incubation time between 30 min and 3 hr may be employed. The reaction was then stopped by the addition of 5 μl of 0.2M EDTA, pH8.0 followed by heat treatment at 100°C for 5 min. To remove unincorporated nucleotides, the labeled probes were purified by passing through NucTrap® push columns (Stratagene).

3.3.5 Purification of the Positive Clones

Fourteen positive clones were picked from the NZY agar plates of primary screening and resuspend in 0.5 ml SM (Section 3.2) with 5μl chloroform added. They were shaken at room temperature for 3 hr. After centrifugation to remove cell debris and agar, the supernatants containing the phage were stored at 4°C. These phage stocks were then diluted 1000X. About 100 p.f.u. was plated out on NZY agar plates (90 mm in diameter) with 200 μl OD_{600}=0.5 host cell/plate and 2.5 ml NZY top agar/plate. The plates were incubated in 37°C until the plaque with a diameter of about 2mm. Filters lifting and hybridization with the same cDNA probe at the same hybridization condition as described in Section 3.3.3 were repeated until all plaques showed positive signals. Ten isolated plaques (P1 to P10) with positive signals were

picked and resuspend in 0.5ml SM with 5µl chloroform after a total of three more rounds of screening (i.e. until plaque-purified).

3.3.6 Purification of DNA from Lambda Phage Using Sephaglas™ PhagePrep Kit (Pharmacia)

The 10 positive clones, each contain 10^5 to 10^6 phages, were plated on 150 mm NZY agarose plates in an NZY soft agarose mix containing appropriate bacterial host cells for phage infection separately. Over a 6 to 10 hr period, the plaques from each individual phage enlarged and became confluent, effectively lysing most of the host cells. After confluent lysis was achieved, the soft agarose layer was harvested by scraping with a bent Pasteur pipette into a 50 ml polypropylene tube. The plate was rinsed with 8 ml SM buffer (Section 3.2) and the buffer was pooled with the scraped agarose. Seven hundred µl chloroform was then added to the mixture and incubated with vigorous shaking for 15 min at 37°C. The agarose was separated from solution containing phage by centrifugation at 6,000 rpm for 10 min at 4°C. About 8 ml supernatant was decanted from the agarose pellet and was ready for preparation of phage DNA.

Using the Sephaglas™ PhagePrep Kit (Pharamica), the 8 ml phage supernatant was mixed with 2 ml DEAE Sephacel provided by the kit and incubated for 3 min at room temperature with gentle rocking to keep the matrix suspended. Then, the tube was centrifuged at 1,000 x g for 5 min and the supernatant was transferred to a new tube to remove bacterial DNA and RNA. This DEAE treatment step was repeated twice. Eighty µl of glacial acetic acid was then added to the supernatant. The tube was vortexed and incubated at room temperature for 2 min. The addition of acetic acid to the supernatant caused the phage to aggregate and bind efficiently to the Sephaglas

PH, provided by the kit, added later. Then, 800 µl of Sephaglas PH were added to the phage/acetic acid mixture. The tube was vortexed gently every min and incubated for 5 min at room temperature. The Sephaglas was then pelleted by centrifugation at 1,000 x g for 3 min and the supernatant was carefully removed. The Sephaglas was then resuspend by adding 1 ml of Binding Buffer (Section 3.2) and transferred to minicolumn provided by the kit. The column was placed in 12 x 75 mm centrifuge tube and then spun at 300 x g for 3 min to remove the supernatant. This wash step was repeated once to remove the protein and help binding of DNA to the Sephaglas. The Sephaglas pellet was spun briefly and allowed to air dry for 10 min to remove any residual ethanlol. Finally, the DNA was eluted by 400 µl preheated water (55-60°C). The absorbance of the DNA at 260 nm and 280nm was measured to detect the yield of DNA.

3.3.7 Restriction Enzyme Digestion Relaese of Inserts

One µg of phage DNA of each of the clones was digested with 10 units of *Xba* I, *Xba* I + *Eco* RI, and *Xba* I + *Hin*d III for 4 hr at 37 °C in approximate buffer separately. *Xba* I restriction enzyme cut at the restriction sites of the multiple cloning site of the Lambda FIX® II vector flanking the 5' end and 3' end of the genomic DNA insert and the insert itself. Agarose gel electrophoresis was performed and the digested DNA was fractionated on 1% (w/v) 1X TAE agarose gel.

3.3.8 Capillary Transfer of DNA to Nylon Membrane Under Alkaline Condition

After agarose gel electrophoresis, the DNA in the gel was denatured by soaking the gel in the denaturing solution (Section 3.2) for 45 min with constant

gentle agitation. A piece of nylon membrane (Hybond N, Amersham) was cut to the exact size as the gel. A sheet of 3MM paper was cut to the same width as the gel but long enough to form a bridge between the buffer reservoir of the transfer. It was wetted by the alkaline transfer buffer (Section 3.2). The gel was then removed from the denaturing solution and inverted so that its underside faced upward. The wet nylon filter which has been soaked in the alkaline transfer buffer was placed onto the gel. Any air bubble between the membrane and the gel was removed. Three wet 3MM papers were placed on the top of the nylon filter and any air bubble trapped was smoothed out by a glass rod. A stack of paper towels (5-8cm high) was then placed on the top of the 3MM papers. A glass plate was put on the top of the stack and a 500g weight was placed on the top (Sambrook *et al.*, 1989).

3.3.9 Southern Analysis of the 10 Positive Clones

The IGF1P insert (obtained from Chapter 2) containing the 5'-flanking region of common carp IGF-I gene, together with the following common carp IGF-I cDNA fragments: the *Rsa* I fragment encoding for a part of signal peptide and the B, C, A, D domains, and the *Xho* Ib fragment encoding for part of the E domain and the 3'-UTR (Liang *et al.*, 1996) were prepared as described in Section 2.3.5. These three probes were labelled with α^{32}P-dCTP using an Amersham nick translation kit (Section 3.3.4). The blots were first hybridized with the *Rsa* I fragment. The blots were hybidized and washed at the same hybridization condition as described in Section 3.3.3, and exposed to Kodak BIOMAX^{TM} MR film at -70^{o}C for 1 to 4 days between intensifying screens. The same blots were then washed twice in 100^{o}C water for 10 min to wash away the radioactive probe, and exposed to Kodak BIOMAX^{TM} MR film

at -70°C for 10 days to ensure no radioactive probe remain in the blot. The blots were then hybridized with the *Xho* Ib fragment and then with the IGF1P insert, and washed under the same condition as described above. The blots were then exposed to BIOMAX™ MR film at -70°C for 1 to 4 days.

3.3.10 Restriction Mapping of the Clone P1

After Southern analysis, 1 out of the 10 clones was found to contain the entire gene, P1. Several restriction enzymes (include Ava II, *Eco* RI, *Hin*d III, *Kpn* I, *Nco* I, Sma I, Sst I, *Xba* I) were used to digest the P1 clone and the condition was the same as in Section 3.3.7. Agarose gel electrophoresis was performed and the digested DNA was fractionated on 1% (w/v) 1X TAE agarose gel. Capillary transfer of DNA to Nylon membrane (Amersham) under alkaline condition (Section 3.3.8) and Southern analysis using the same three probes as described in Section 3.3.9 were carried out.

3.3.11 Subcloning of the Fragments of the Clone P1 into Plasmid Vector

The fragments released from the *Hin*d III restriction enzyme digestion were excised from the agarose gel. Gene Clean (Section 2.3.5) was performed to purify the DNA fragements. The eluted DNA fragments were ligated to *Hin*d III cut and dephosphorylated pUC18 vectors (Pharmacia). After ligation, the vectors were transformed into DH5α competent cells as described in Section 2.3.7.

3.3.12 IGF-I Specific PCR

Fifty ng of clone P1 and 100μg of carp genomic DNA (prepared from Section 2.3.1) were used for PCR, using the Perkin Elmer GeneAmp 9600 Thermocycler and

the Expand™ Long Template PCR System (Boehringer Mannheim). The cycle profile is given as below:

1X	93°C	2 min
10X	93°C	10 s
	55°C	30 s
	68°C	15 min
20X	93°C	10 s
	55°C	30 s
	68°C	15 min
	+ cycle elongation of 20 s for each cycle	
1X	68°C	7 min

In each round of PCR, 2.5 units of an enzyme mixture of *Taq* and *Pwo* DNA polymerases (Boehringer Mannheim) were used and 15 pmole of each primer were used in a reaction of 50 µl. IGF-I specific primers were designed from the starting of common carp cDNA signal peptide and the end of common carp E domain. The relative position of the primers could be seen in Fig. 3.1. Both regions are in fact conserved in the four form of IGF-I mRNAs in chinook salmon (Wallis and Devlin, 1993). The nucleotide sequences of primer set for carp IGF-I is :

IGF-5' primer: 5'GATGTCTAGCGGACATTTCTTC3', and

IGF-3' reverse primer: 5'CTAAATGCGATAGTTTCTTCCC3'.

Ten µl of PCR products were fractionated in a 1% (w/v) 1X TAE agarose gel. After EtBr staining, the samples were transferred onto Hybond-N nylon membrane (Amersham) by capillary alkali-transfer overnight (Section 3.3.8) and Southern analysis using the common carp IGF-I cDNA *Rsa* I probe as described in Section 3.3.9 was carried out.

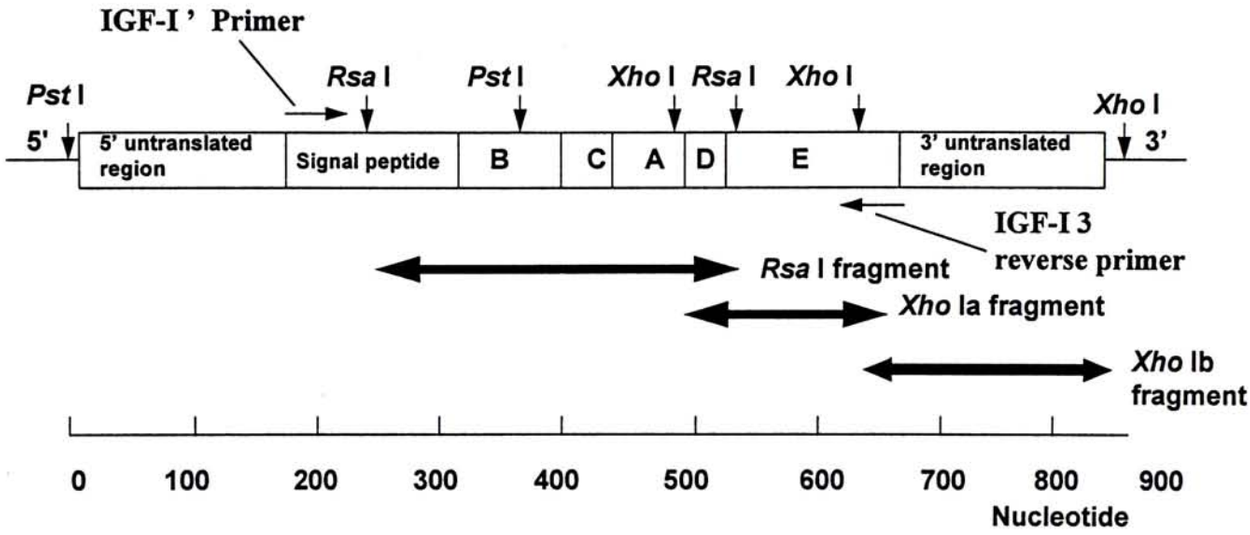

Fig. 3.1 The relative positions of the *Rsa* I fragment, *Xho* Ib fragment and IGF-I specific primers in the common carp IGF-I Ea2 cDNA (Liang *et al.*, 1996).

3.3.13 Amplification of Introns from the Clone P1 Using PCR

According to the structure of salmon IGF-I gene (Kavsan *et al.*, 1993), primers were designed to amplify introns from the clone P1 using PCR. Fifty ng of clone P1 and 100μg of carp genomic DNA (prepared from Section 2.3.1) were used for PCR, using the Perkin Elmer GeneAmp 9600 Thermocycler and the Expand™ Long Template PCR System (Boehringer Mannheim). The cycle profile is given as below:

1X	93°C	2 min
10X	93°C	10 s
	55°C	30 s
	68°C	4 min
20X	93°C	10 s
	55°C	30 s
	68°C	4 min
	+ cycle elongation of 20 s for each cycle	
1X	68°C	7 min

In each round of PCR, 2.5 units of an enzyme mixture of *Taq* and *Pwo* DNA polymerases (Boehringer Mannheim) were used and 15 pmole of each primer were used in a reaction of 50 μl. The position of the primer could be seen in Fig. 3.2. The nucleotide sequences of primer sets for amplification of introns are:

Intron 1,

5' primer (IG2)	5'ATGTCTAGCGGACATTTCTTCCA3', and
3' reverse primer (IG3)	5'ACGAGAGACAGCGCATGGTACA3';

Intron 2,

5' primer (IG4)	5'GAGACAGGGGCTTTTATTTCA3', and
3' reverse primer (IG5)	5'CGTCTTCAACTAGGCCCATAT3';

Intron 3,

5' primer (IG6)	5'CCCGTAAAGCCCGGCAAAACT3', and

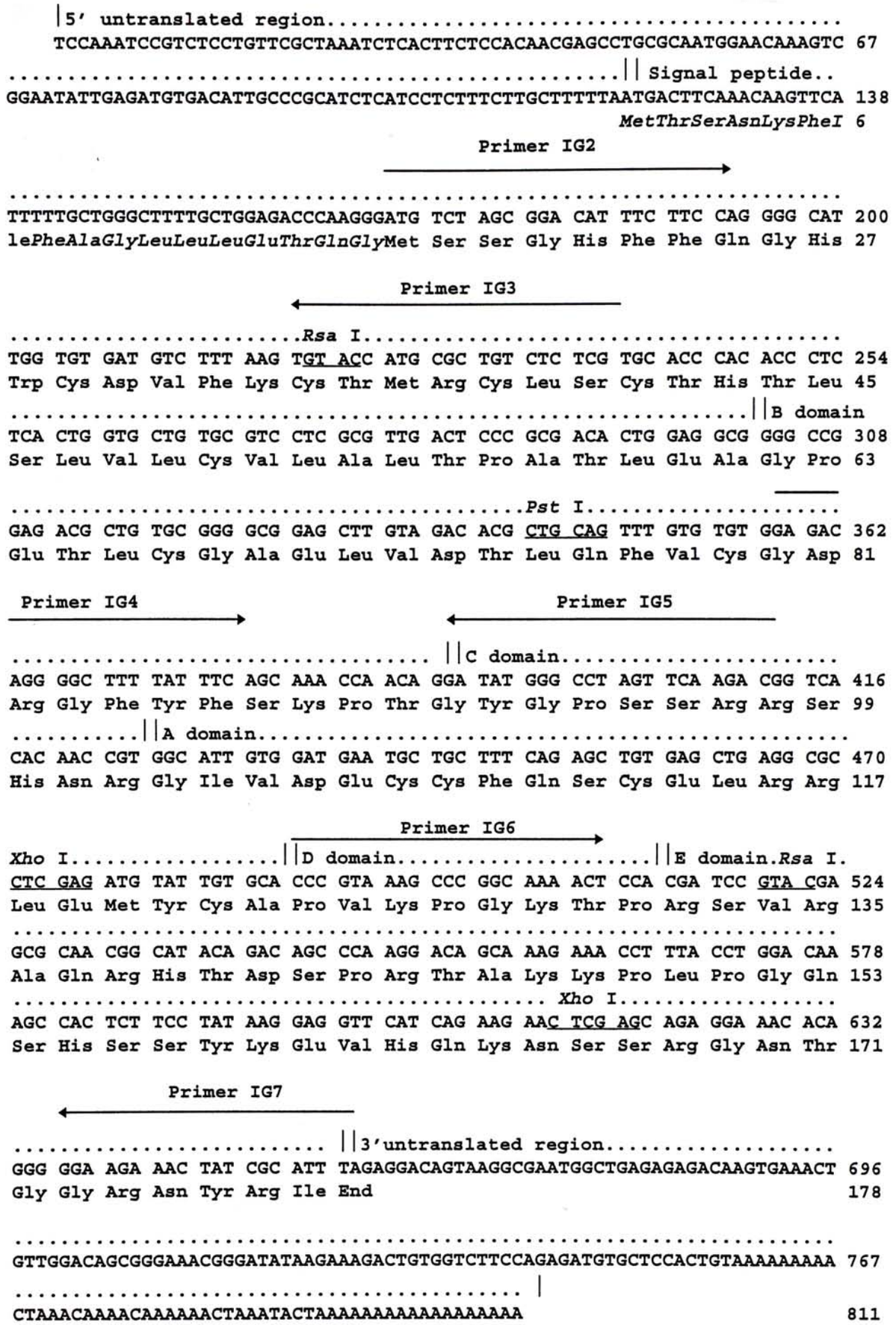

Fig. 3.2 Nucleotide sequence and the predicted amino acid sequence of the common carp IGF-I cDNA clone (Liang *et al.*, 1996). Labeled arrows above the sequence represent the positions and 5' to 3' direction of primers used in PCR for amplification of introns.

3' reverse primer (IG7) 5'CTAAATGCGATAGTTTCTTCC3'.

Ten μl of PCR products were fractioned in a 1% (w/v) 1X TAE agarose gel. The PCR products were cloned into pCR® 2.1 vector as described in Section 2.3.6. DNA sequencing was performed using the Universal primer and Reverse primer as described in Section 2.3.11.

3.4 Results and Discussion

For the primary screening of 600,000 recombinant bacteriophages of the common carp genomic library on twelve plates with the *Rsa* I fragment of the common carp of IGF-I cDNA probe, we initially identified 14 positive signals after autoradiography, as shown in Fig. 3.3. In the plaque purification steps, ten plaque-purified clones with positive signals were picked and named as P1 to P10 as shown in Fig. 3.4.

In order to release the inserts from the phages, *Xba* I was used because this restriction enzyme cut the restriction sites at the multiple cloning site of the Lambda Fix II vector flanking the 5' end and 3' end of the genomic insert. The long arm and short arm of the phage vector were released as 22 kb and 9 kb DNA fragments respectively. The inserts of the 10 plaque-purified clones were released as several *Xba* I cut fragments. Double restriction-enzyme digestion, using either *Eco* RI or *Hind* III restriction enzyme in combination with *Xba* I restriction enzyme, were also used to characterize these 10 clones (Fig. 3.5 and 3.6). Then, Southern blot analysis was carried out to identify the released fragments. The probes used were: (1) 5'-flanking region of the common carp IGF-I gene (clone IGF1P); (2) *Rsa* I fragment of common carp IGF-I cDNA (which contains the B, C, A and D domains coding for the mature IGF-I peptide); (3) *Xho* Ib fragment of common carp IGF-I cDNA (which contains the 3'-UTR of the IGF-I gene) (Fig. 3.5 and 3.6) According to the difference in restriction pattern, there are mainly two types of clones. One type of clones such as P1, P4, P7 and P8 with similar restriction pattern, and another type of clones such as P2, P3, P5 and P6 with different pattern. From these preliminary results, we found that only clone P1 could hybridize to the three probes used in Southern blot analysis and other clones could hybridize only to one or two of the three probes. Therefore, clone P1 probably

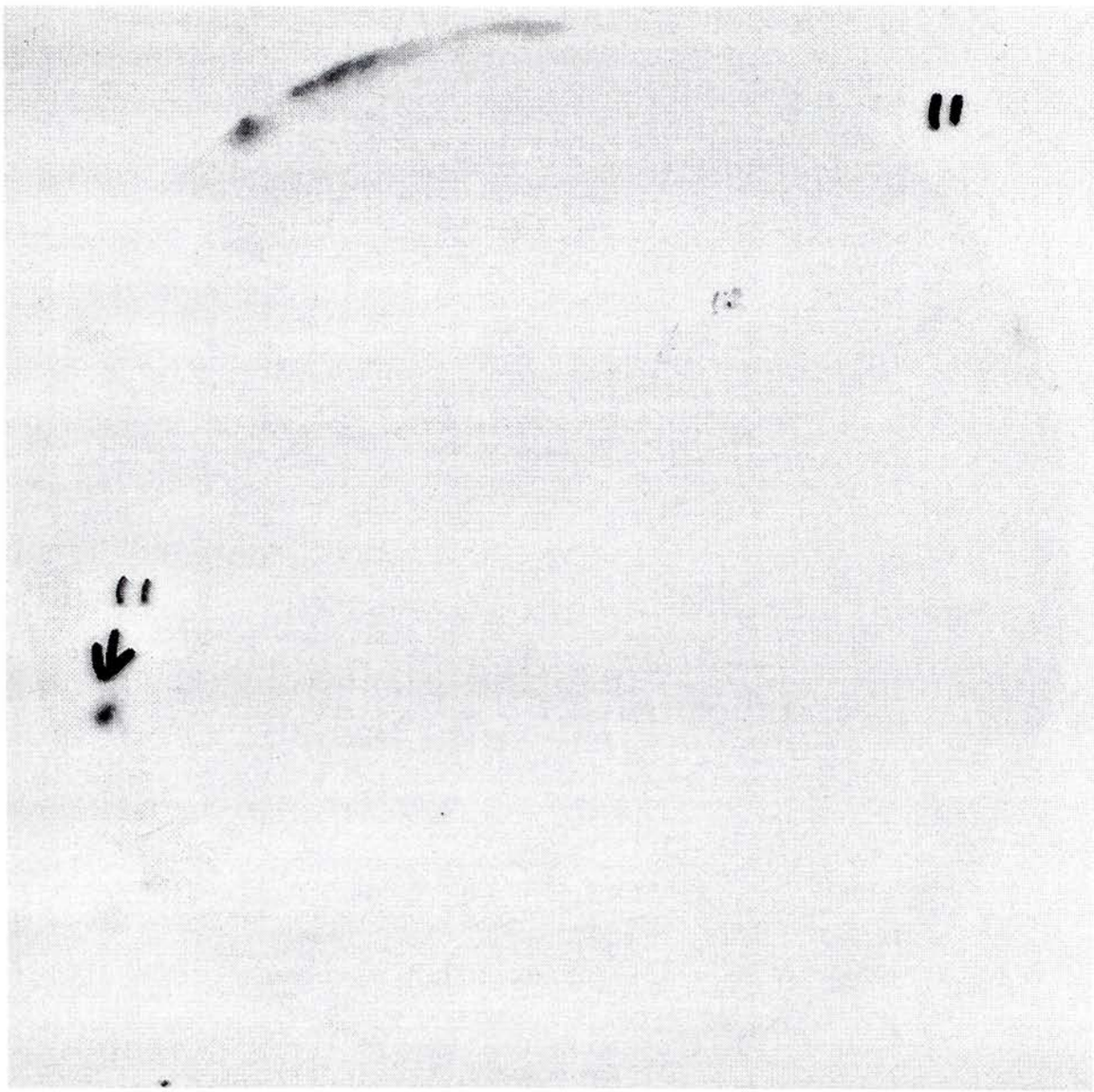

Fig. 3.3 An autoradiograph showing a positive signal on one of the filter liftsfrom a primary screening of common carp genomic library using common carp IGF-I cDNA conserved region, *Rsa* I fragment (which contains the B, C, A and D domains coding for mature IGF-I peptide). The arrow point to the position of one of the positive signal, temporary labeled as #11.

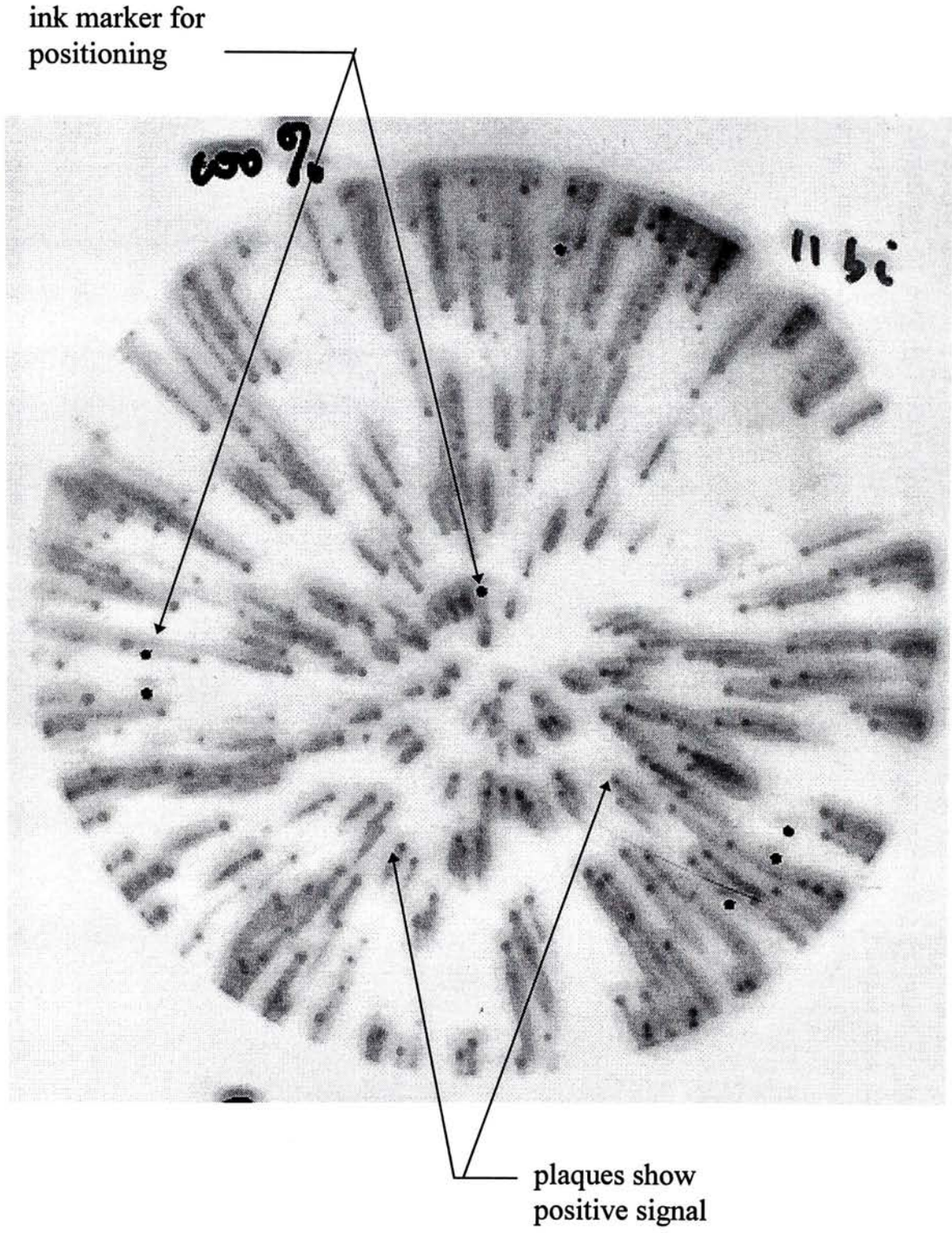

Fig. 3.4 An autoradiograph showing the positive signals from one of the nylon filter after the plaque purification steps. This clone corresponds to clone #11 in the primary screening.

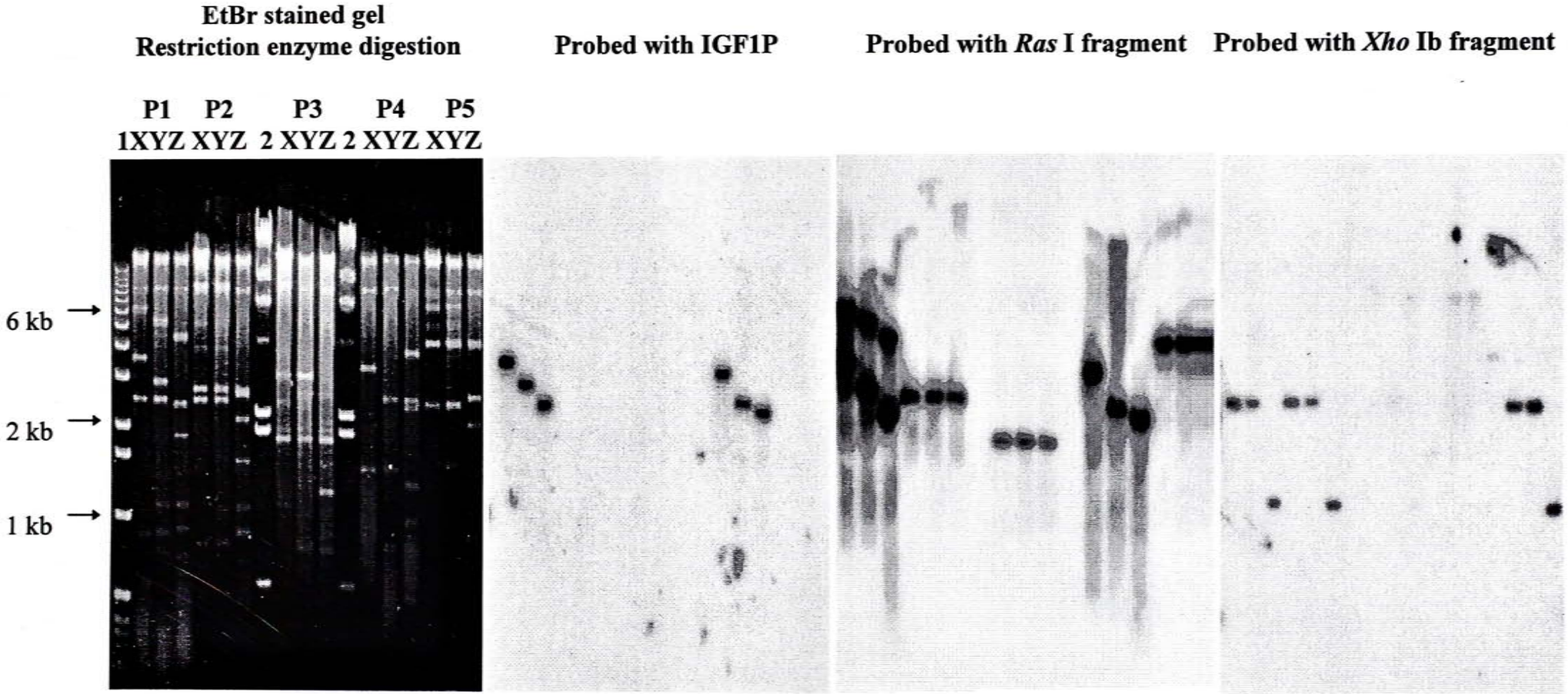

Fig. 3.5 Restriction enzyme digestion and Southern blot analysis of positive clones (P1 to P5) obtained from primary screening of common carp genomic library. The probes used were: (1) 5'-flanking region of common carp IGF-I gene (IGF1P); (2) *Rsa* I fragment of common carp IGF-I cDNA (which contains the B,C, A and D domains coding for mature IGF-I peptide); (3) *Xho* Ib fragment of common carp IGF-I cDNA (which contains the 3'-UTR of IGF-I gene). The corresponding restriction enzymes used in each lane are labeled as X (*Xba* I), Y (*Xba* I + *Eco* RI) and Z (*Xba* I + *Hin*d III). Lane 1 was loaded with 1 kb size marker whereas Lane 2 was loaded with λ *Hin*d III size marker.

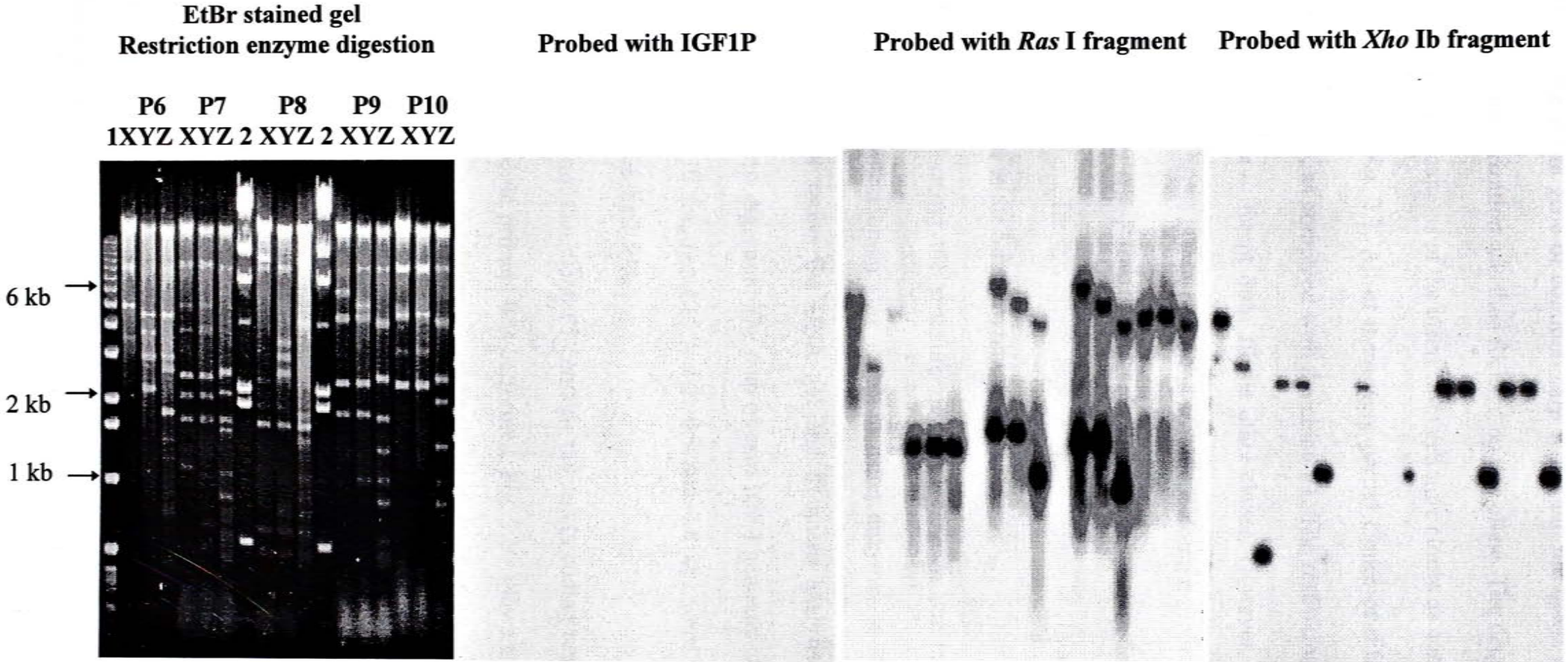

Fig. 3.6 Restriction enzyme digestion and Southern blot analysis of positive clones (P6 to P10) obtained from primary screening of common carp genomic library. The probes used were: (1) 5'-flanking region of common carp IGF-I gene (IGF1P); (2) *Rsa* I fragment of common carp IGF-I cDNA (which contains the B,C, A and D domains coding for mature IGF-I peptide); (3) *Xho* Ib fragment of common carp IGF-I cDNA (whichs contain 3'-UTR of IGF-I gene). The corresponding restriction enzymes used in each lane are labeled as X (*Xba* I), Y (*Xba* I + *Eco* RI) and Z (*Xba* I + *Hin*d III). Lane 1 was loaded with 1 kb size marker whereas Lane 2 was loaded with λ *Hin*d III size marker.

contain the entire gene of common carp IGF-I and the insert size is about 13 kb. Our further characterization was then focused on this clone. The diagrammatic representation of the relative positions of the insert from genomic clones as compared with the clone P1 was shown in Fig. 3.7. All of them were partial clones of different parts of the IGF-I gene, except P6, as compared with the clone P1. The restriction pattern of the clone P6 was found to be different from all the other clones. However, it binds to the common carp conserved IGF-I cDNA probe and specific E-domain probe. Thus, this clone may be a subtype of the IGF-I gene. To find out the answer, nucleotide sequence determination may be carried out for this clone, especially the fragments hybridized with the specific E-domain probe in the Southern blot analysis.

To understand the structure of IGF-I gene in common carp, clone P1 was subjected to restriction-enzyme mapping using enzymes as described in the Section 3.3.10. Fig. 3.8 shows the DNA gel and Southern analysis of the clone P1 after restriction enzyme digestion. The map of carp IGF-I genomic clone P1 is shown in Fig. 3.9. The *Hind* III fragments (2.7, 1.1, 0.9 and 0.8 kb) were subcloned into pUC 18 vector, except for the 5.5 kb.

To confirm that clone P1 contains the whole coding region of IGF-I gene. PCR using IGF-I specific primers was performed. Fig. 3.10 shows the PCR products formed using the IGF-I 5' and IGF-I 3' reverse primer. The template of the positive control was the common carp genomic DNA. A sharp band of ~11 kb was amplified from the clone P1 so clone P1 probably contained the entire IGF-I gene. However, a smaller (~10 kb) DNA fragment was amplified using common carp genomic DNA as template, probably because different strains of common carp was used. The common carp used for genomic DNA preparation was obtained from local market but the library was obtained from Stratagene, La Jolla, using carp from USA. Southern blot study of the IGF-I specific

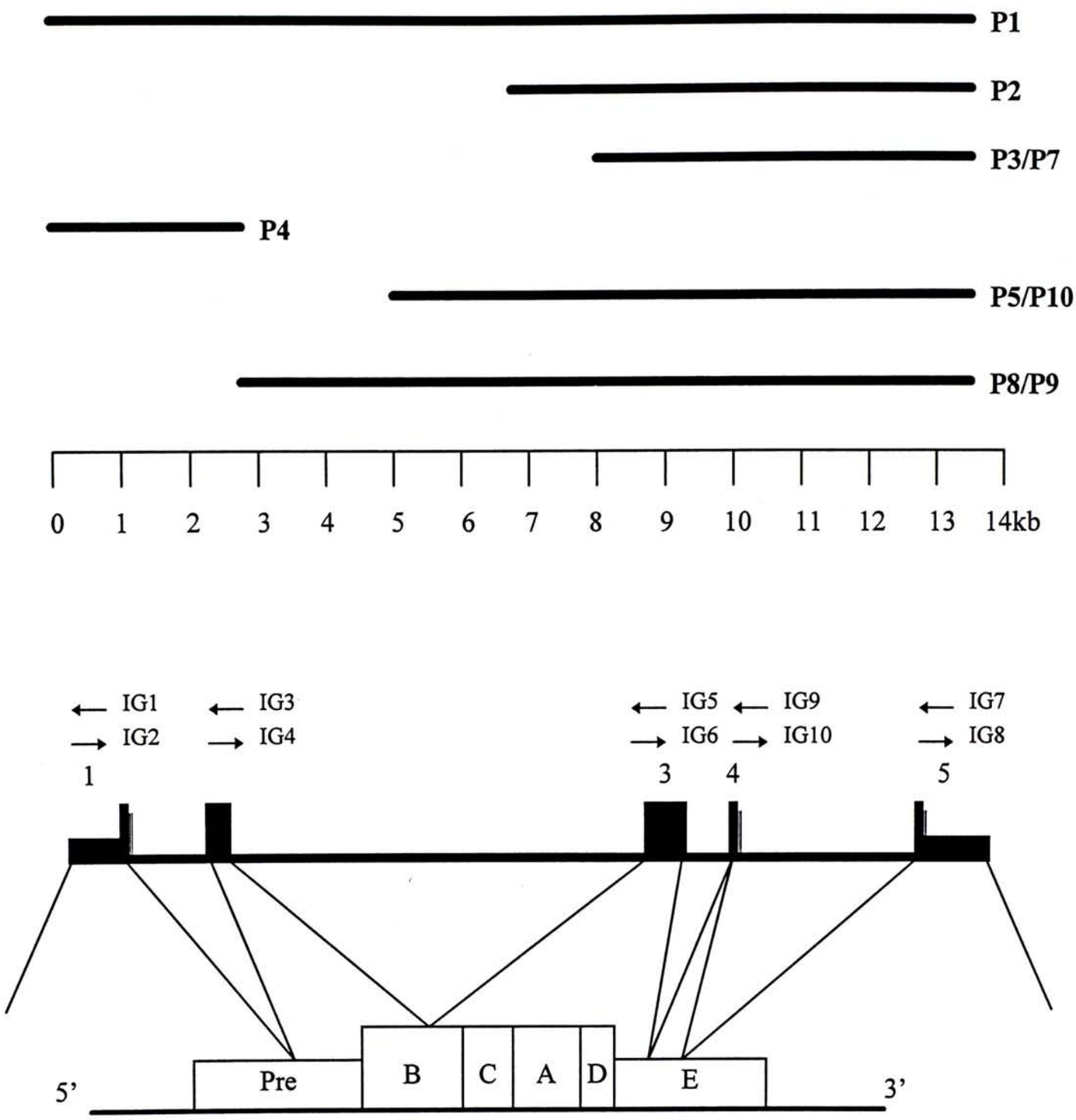

Fig. 3.7 Diagrammatic representation of the relative positions of the inserts from clones P1, P2, P3, P4, P5, P7, P8, P9 & P10. The solid lines shown above the scale bar represent the relative positions of the clones. Labeled arrows represent the relative position and 5' to 3' direction of the primers used in Takara PCR and DNA sequencing. Exons are deplicted by boxes (not in scale), and introns and flanking regions by the lines (in scale).

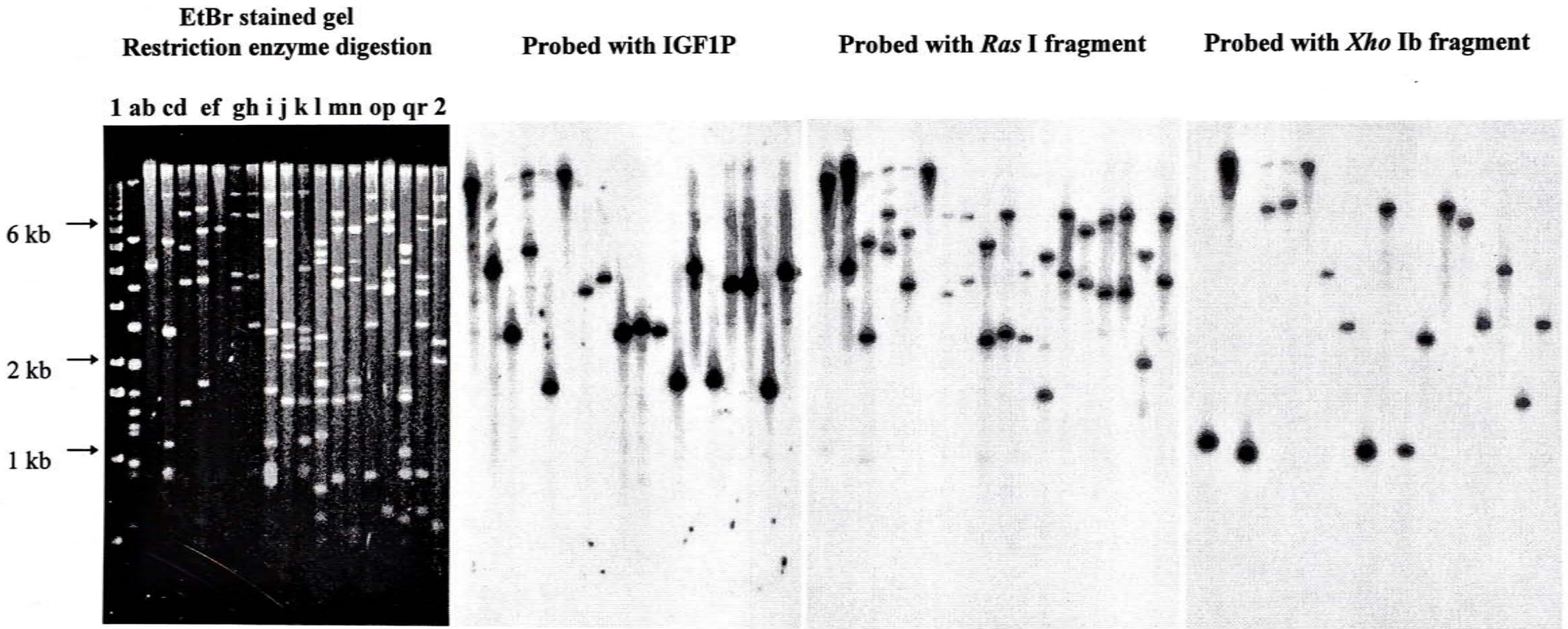

Fig. 3.8 Restriction enzyme digestion and Southern blot analysis of positive clone P1. The probes used were: (1) 5'-flanking region of common carp IGF-I gene (IGF1P); (2) *Rsa* I fragment of common carp IGF-I cDNA (which contain the B,C, A and D domains coding for mature IGF-I peptide); (3) *Xho* Ib fragment of common carp IGF-I cDNA (which contain 3'-UTR of IGF-I gene). The corresponding restriction enzymes used in each lane are labeled as a (*Ava* II), b (*Eco* RI), c (*Hind* III), d (*Kpn* I), e (*Nco* I), f (*Sma* I), g (*Sst* I), h (*Xba* I), i (*Eco* RI + *Hin*d III), j (*Eco* RI + *Kpn* I), k (*Hin*d III + *Kpn* I), l (*Kpn* I + *Sma* I), m (*Kpn* I + *Sma* I), n (*Nco* I + *Sma* I), o (*Nco* I + *Sma* I), p (*Sst* I + *Sma* I), q (*Xba* I + *Nco* I), and r (*Xba* I + *Sma* I). Lane 1 was loaded with 1 kb size marker whereas Lane 2 was loaded with λ *Hin*d III size marker.

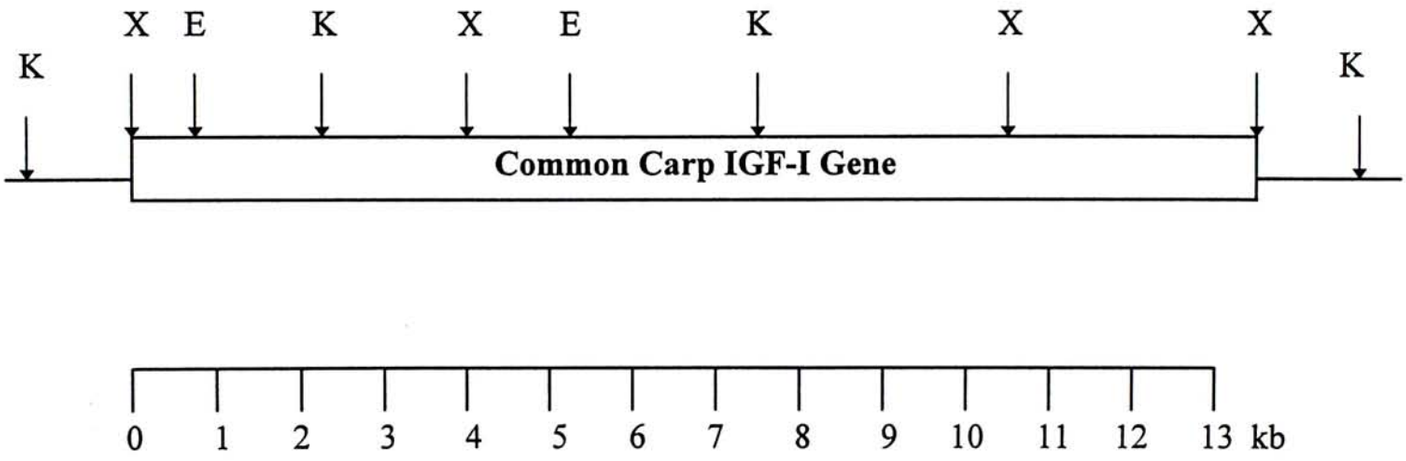

Fig. 3. 9 Map of common carp IGF-I gene. Open box represents the phage clone P1 covering 13.5 kb genomic region. The restriction enzyme sites shown are E, *Eco* RI; K, *Kpn* I; X, *Xba* I.

Lane 1 -- 1kb marker
Lane 2 -- Clone P1 + Primers I5 and I3
Lane 3 -- Clone P1 + Primer I5
Lane 4 -- Clone P1 + Primer I3
Lane 5 -- Water + Primers I5 and I3
Lane 6 -- Common carp genomic DNA + Primers I5 and I3
Lane 7 -- Common carp genomic DNA + Primer I5
Lane 8 -- Common carp genomic DNA + Primer I3

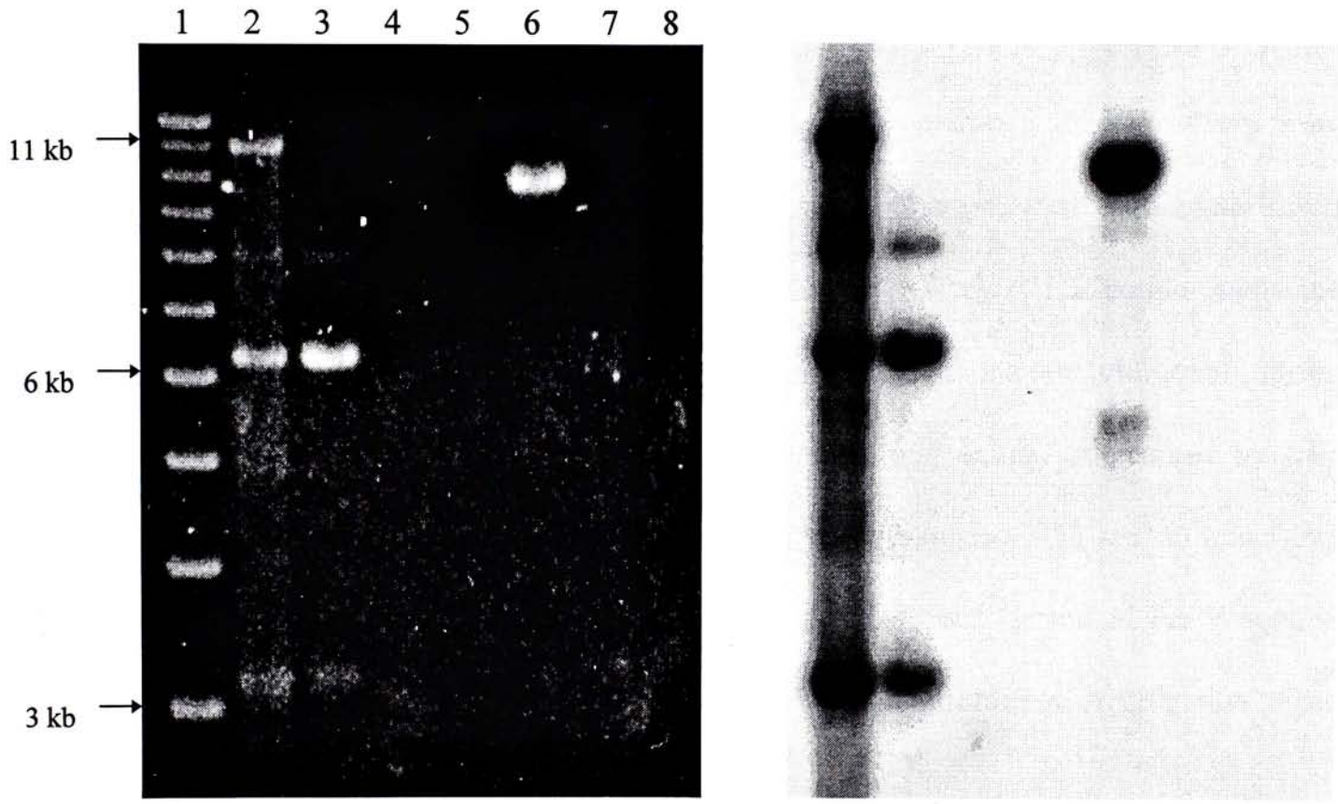

Fig. 3.10 Left panel: The DNA gel (after EtBr staining) showing the PCR products of IGF-I specific PCR using IGF-I 5' primer (I5) and IGF-I 3' reverse primer (I3). The template used is either clone P1 and common carp genomic DNA. Right panel: The Southern blot analysis of the PCR products using the *Rsa* I fragment of the cloned common carp IGF-I Ea2 cDNA as a probe for hybridization.

PCR products using *Rsa* I fragment of common carp IGF-I Ea2 cDNA as a probe is also shown in Fig. 3.10.

The chum salmon IGF-I gene contains three introns (Kavsan *et al.*, 1993). Based on the structure of chum salmon IGF-I gene and the sequence of the common carp IGF-I cDNA, three pairs of primers were designed to amplify the introns from the clone P1 using PCR (Fig. 3.11). Intron I was found to be 1.2 kb in size, intron II was found to be about 6.5 kb in size and intron III was found to be about 3.2 kb in size. We also found that the size of intron III using common carp genomic DNA as template is about 1.3 kb less than that obtained using clone P1 as template, confirming the strains difference between local carps and American carp. Then, the introns were cloned into the pCR® 2.1 vector and the intron/exon junctions were sequenced. Intron I and III were successfully sequenced. However, after nucleotide sequence determination, we found that intron III amplified contains an additional intron. Therefore, the common carp IGF-I gene contains five exons, interrupted by four introns. This structural organization was found to be similar to the second non-alleic IGF-I gene of chum salmon which contains one additional intron in the E-peptide region (Kavsan *et al.*, 1994). Fig. 3.12 summarizes the intron-exon organization of the common carp IGF-I gene.

However, we only determined the intron/exon junctions of the cloned IGF-I gene. To understand the entire structure and confirm whether the clone belong to IGF-I Ea2 subtype, nucleotide sequence determination may be carried out for the subcloned *Hind* III fragments.

Lane 1 -- Clone P1 + Primers IG2 and IG3
Lane 2 -- Clone P1 + Primers IG4 and IG5
Lane 3 -- Clone P1 + Primers IG6 and IG7
Lane 4 -- Water + Primers IG2 and IG3
Lane 5 -- Water + Primers IG4 and IG5
Lane 6 -- Water + Primers IG6 and IG7
Lane 7 -- Common carp genomic DNA + Primers IG2 and IG3
Lane 8 -- Common carp genomic DNA + Primers IG4 and IG5
Lane 9 -- Common carp genomic DNA + Primers IG6 and IG7
Lane 10 -- 1 kb marker

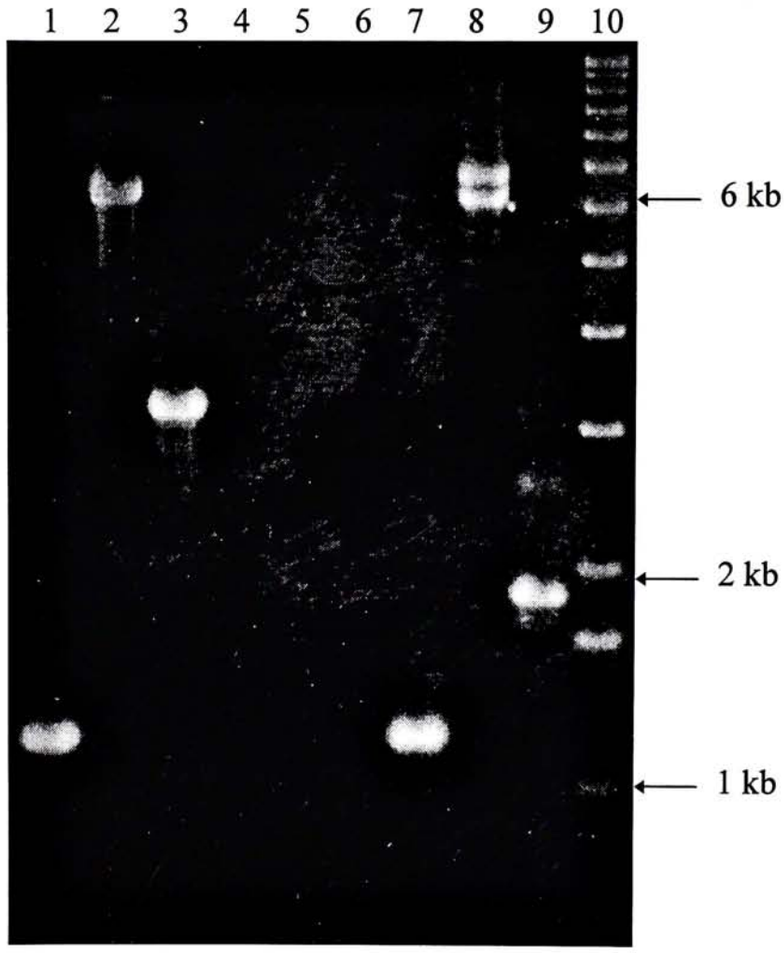

Fig. 3.11 The DNA gel (after EtBr staining) showing the PCR products from the amplification of introns using IGF-I gene specific primers designed from the common carp IGF-I Ea2 cDNA. The template used is either clone P1 and common carp genomic DNA.

Exon	Exon size bp	5' Splice donor	Intron size kb	3' Splice acceptor
1	~1k	GTCTTTAAGgtaac... ValPheLys	1.2	..tgcagTGTACCATG...exon 2 CysThrMet
2	160	TTTTATTTCAgtaag.. PheTyrPheS	6.5	..aacagGCAAACCAACA.exon 3 erLysProThr
3	182	ACAGCAAAGgtgtg... ThrAlaLys	0.7	..tacagAAACCTTTA...exon 4 LysProLeu
4	36	To be determined	2.5	..atcagGAGGTTCAT...exon 5 GluValHis
5	~1k	-	-	-

Fig. 3.12 Intron-exon organization of the common carp IGF-I gene.

CHAPTER 4 RNA ASSAY USING REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

4.1 Introduction

The basic mechanism of GH-IGF axis is also shown to be operative but in a different manner in teleost. For example, both IGF-I and GH were found to be related to somatic growth in fish (Skyrud *et al.*, 1989; Gray and Kelly, 1991; McCormick *et al.*, 1992). In rainbow trout, however, both IGF-I and IGF-II also show GH-dependence (Shamblott *et al.*, 1995).

RNase protection assay was used to study the effect of bovine GH on the levels of IGF-I and IGF-II mRNA in juvenile rainbow trout (*Oncorhynchus mykiss*) (Shamblott *et al.*, 1995). A single i.p. injection of bovine GH at a dose of 10 ug/g body weight resulted in a significant increase in IGF-I and IGF-II mRNA levels in liver when compared with control. In another experiment, homologous radioimmunoassay was also used to study the effect of salmon GH on the level of plasma IGF-I in rainbow trout (*Oncorhynchus mykiss*) (Moriyama, 1995). A single i.p. injection of salmon GH at a dose of 0.1 or 1.0 ug/g body weight resulted in a significant increase in plasma IGF-I level in a dose-dependent manner.

Since both IGF-I and IGF-II mRNA are found in adult and juvenile fish tissues, it was suggested that both IGF-I and IGF-II may have somatic growth effects in both juvenile and adult trout (Shamblott and Chen, 1993). Whether this phenomenon is universally true for other fish species remains to be established.

Amplified PCR products increase exponentially with reaction cycle, therefore, even a slight increase in the number of initial templates will drastically increase the efficiency of PCR. Reverse transcriptase (RT), capable of generating multiple copies of cDNA from mRNA prior to PCR, greatly increase the amplification products and

enhances sensitivity and specificity of analysis. It also provides an opportunity to study multiple gene expression (Liu *et al.*, 1997).

Using RT-PCR, it was found that IGF-I Ea2 is the predominantly expressed form of IGF in the liver of adult common carp (Liang *et al.*, 1996). In this project, by visualizing the gene products after ethidium bromide staining, we used a semiquantitative RT-PCR method to investigate the effect of fish GH on the level of hepatic IGF-I mRNA in common carp. The total RNA of liver was prepared and RT-PCR using IGF-I specific primers was performed.

4.2 Materials

Growth hormone (Section 4.3.1)
The fish growth hormone used in the present study is the recombinant black bream GH obtained commerically from GroPep, Australia.

Saline (Section 4.3.1)
0.9% NaCl
Sterilized by autoclaving for 20 min at 15lb/sq. in. liquid cycle.

DEPC-H_2O (Section 4.3.2, 4.3.3 and 4.4.4)
Add diethyl pyrocarbonate (DEPC) to 0.1% in ddH_2O and stir overnight, then sterilize the solution by autoclaving for 20 min at 15lb/sq. in. liquid cycle.

5X formaldehyde gel-running buffer (Section 4.3.3)
0.1 M MOPS, pH 7.0
40mM sodium acetate
5mM EDTA

Formaldehyde gel-loading buffer (Section 4.3.3)
50% glycerol
1mM EDTA
0.25% bromophenol blue
0.25 % xylene cyanol FF

20X SSC (Section 4.3.4)
175.3g NaCl
88.2g sodium citrate
Add ddH_2O to a final volume 1 liter, adjust pH to 7.0 with 10N NaOH, treated with diethyl pyrocarbonate (DEPC) (0.1% in water) and stir overnight, then sterilize the buffer by autoclaving for 20 min at 15lb/sq. in. liquid cycle.

10% SDS (Section 4.3.4)
100g SDS
Add ddH_2O to 1 liter, heat to 68°C, adjust pH to 7.2.

4.3 Methods

4.3.1 Adminstration of Hormones

Two injection time courses were used in common carp IGF-I gene expression studies. The sbGH (Section 4.2)was dissolved in saline (Section 4.2)

4.3.1.1 Injection Time Course 1

Groups (10 fish per group) of juvenile common carp were transferred to smaller indoor static tanks (10L) with aerated fresh water under natural temperature (~15°C) and photoperiod for 10 days prior to the experiment. They were kept on daily feeding regimen during the experiment. The fish received i.p. injections of 1000, 100 and 0 (saline control) ng sbGH in a volume of 10 μl/g total wet body weight every other day between 9 am and 12 pm for a total of 4 injections (in a span of 7 days). The fish were killed 24 hr after the last injection. Liver was collected and pooled together from each group and immediately transferred to liquid nitrogen. The tissues was then stored at -70 °C until RNA extraction. The expression of IGF-I mRNA was then studied using Northern blot analysis of poly A^+ mRNA with a common carp IGF-I probe (conserved region).

4.3.1.2 Injection Time Course 2

Groups (3 fish per group) of junvenile common carp were transferred to smaller indoor static tanks (10L) with aerated fresh water under natural temperature (~15°C) and photoperiod for 10 days prior to the experiment. Fish were not fed for 24 hr before fGH adminstration. The fish received i.p. injections of 1000, 100 and 0 (saline control) ng sbGH in a volume of 10 μl/g total wet body weight every twelve hr for a total of 2 injections (within 1 day). The fish were killed 12 hr after the last

injection. Liver was collected from each individual and immediately transferred to liquid nitrogen. The tissues was then stored at -70 °C until RNA extraction. The expression of IGF-I mRNA was then studied using RT-PCR.

4.3.2 Total RNA Extraction

Glassware after autoclaved was baked at 150°C overnight. Plasticware was filled with 0.1% DEPC (Section 4.2) after stirring overnight and then autoclaved. Electrophoresis tanks used for electrophoresis of RNA were cleaned with detergent, rinsed in water and then soaked with a solution of 3% H_2O_2. After incubated at room temperature overnignt, the tanks were rinsed with DEPC treated water. Gloves were worn at all times during the experiment because hands of the investigator were potentially major sources of RNase contamination.

4.3.2.1 Rapid RNA Isolation

TRI REAGENT® (Molecular Research Centre, Inc.) was used to extract the total RNA from common carp liver. For one hundred mg tissues, 1 ml TRI REAGENT® was used. The tissues was then homogenized with a tissue grinder. The mixture was stored on ice for 5 min. Then, 0.1 ml bromochloropropane (BCP) was added and mixed vigorously. The mixture was then cooled on ice for 10 min followed by centrifugation at 4°C and 14,000 rpm for 15 min. The upper aqueous phase containing the RNA was transferred to a fresh tube, whereas the DNA and proteins were in the interphase and lower organic phase. Half a ml of 100% isopropanol was added to the aqueous phase to precipitate the RNA. The sample was stored at -20°C overnight followed by centrifugation for 15 min at 14,000g at 4°C. The supernatant

was discarded and the pellet was washed by 1 ml 75% ethanol. It was then centrifuged for 5 min at 14,000g and the supernatant was discarded. The pellet was dried in vacuum for 5 min and then dissolved in 50 µl DEPC-treated water. The absorbance of the RNA at 260 nm and 280nm was measured. The RNA was then stored in -70°C.

4.3.3 Electrophoresis of RNA in Agarose Gel Containing Formaldehyde

The isolated total RNA was electrophoresed (Sambrook *et al.*, 1989) in order to check the integrity of the RNA. An 1.2 % agarose gel was prepared in formaldehyde gel-running buffer (Section 4.2) by melting an appropriate amount of agarose in water, cooling it to 60°C, and formaldehyde was added to give a final concentrations 2.2M. The gel was casted and allowed to set for at least 30 min inside a fumehood. Then the samples were prepared by mixing the following components in a microfuge tube: 5 µl of RNA (10 µg), 2.0 µl of 5X formaldehyde gel-running buffer, 3µl of formaldehyde, 10µl of formamide and 1µl of EtBr (1µg/µl). The samples were incubated for 15 min at 68°C, and then quick-chilled on ice for 5 min. Two µl of formaldehyde gel-loading buffer (Section 4.2) was added before loading. As formaldehyde vapors were toxic, solutions containing formaldehyde were prepared in a chemical hood, and electrophoresis tanks containing formaldehyde solution were kept covered whenever possible. The gel was immersed in 1X formaldehyde gel-running buffer. The RNA samples were loaded into the wells of the gel. Electrophoresis was carried out at 70V constant voltage. The RNA in the gel was visualized and photographed by UV illumination.

4.3.4 Rapid Isolation of PolyA$^+$ mRNA from Total RNA

The pooled total RNA samples obtained from injection time course 1 (Section 4.3.1.1) were used as starting materials for underwent polyA$^+$ mRNA isolation using PolyAtract mRNA Isolation System (Promega). One mg of total RNA to a final volume of 500 µl in DEPC-H_2O (Section 4.2) was heat denatured at 65°C for 10 min. Six µl of the Biotinylated-Oligo(dT) probe and 90 µl of 20X SSC (Section 4.2) were added to the RNA. The mixture was mixed gently and incubated at room temperature until completely cooled to allow the annealing of Biotinylated-Oligo(dT) probe (provided by the kit) to the polyA$^+$mRNA. Then, the entire contents of the annealing reaction was added to the tube containing the washed Streptavidin-Paramagnetic Particles (SA-PMPs) (provided by the kit) and incubated at 37°C with shaking. The SA-PMPs containing the annealed oligo(dT)-mRNA hybrid were captured using the Magnetic Stand provided by the kit. Then, the particles were washed three times with 0.6X SSC (Section 4.2) and captured again with the Magnetic Stand. The mRNA was eluted by resuspending in 250 µl of preheated DEPC-H_2O (65°C) and stored at -70°C.

Ten µg of polyA$^+$ mRNA was fractionated using the method mentioned in Section 4.3.3. Capillary transfer of RNA to nylon membrane (Amersham) in 20X SSC (section 4.2) were carried as described in Section 3.3.8 without the denaturing step and the alkaline transfer buffer was replaced with 20X SSC (Section 4.2). Northern analysis using the carp *Rsa* I fragment of IGF-I cDNA and zebrafish β-actin (positive control, Chan, unpublished data) probes was performed as described in Section 3.3.4.

4.3.5 IGF-I Specific RT-PCR

The Titan™ One Tube RT-PCR System (Boehringer Mannheim) and the Perkin Elmer GeneAmp 9600 thermocycler were employed to determine the expression level of IGF-I in common carp after sbGH i.p. injection (samples obtained from injection time course 2, Section 4.3.1.2). The cDNA reaction as well as the PCR were performed with an optimized buffer and enzyme without requirement for addition of reagents between cDNA synthesis and PCR. The one step reaction system system uses AMV for first strand synthesis and the Expand™ High Fidelity enzyme blend, which consist of Taq DNA polymerase and Pwo DNA polymerase, for the polymerase reaction. The cycle profile is given as below:

1X	50°C	30 min
1X	94°C	2 min
10X	94°C	10 s
	55°C	30 s
	68°C	45 s
15X	94°C	10 s
	55°C	30 s
	68°C	45 s
	+ cycle elongation of 20s for each cycle	
1X	68°C	7 min

In each reaction, 1 μg of total RNA, 1 μl of the enzyme mixture, and 20 pmole of each primer were used in a reaction of 50 μl. The position of the primer could be seen in Fig. 3.1. The nucleotide sequences of primer sets for carp IGF-I and actin control are:

Carp IGF-I,

IGF-5' primer 5'GATGTCTAGCGGACATTTCTTC3' and

IGF-3' reverse primer 5'CTAAATGCGATAGTTTCTTCCC3';

Actin control,

Actin-5' primer 5'TCACCAACTGGGATGACATG3' and

Actin-3' reverse primer 5'ATCCACATCTGCTGGAAGGT3'

Ten µl of PCR product were fractionated in a 2% (w/v) 1X TAE agarose gel. The intensity of each band was determined using a PC version of ImageQuaNT (Molecular Dynamics) software in a laser densitometer (Personal Densitometer SI™, Molecular Dynamics).

4.4 Results and Discussion

For the injection time course 1, the liver samples in each group following intraperitoneal injections of sbGH at doses of either 100 or 1,000 ng/g were pooled and total RNA was then purified (Fig. 4.1). Changes in hepatic IGF-I mRNA are shown in Fig. 4.2. Ten μg of polyA$^+$ mRNA were isolated from the total RNA and fractionated using electrophoresis. Northern blot analysis was used to determine the expression level of hepatic IGF-I mRNA. Three bands were identified. Therefore, it may have three mRNA transcripts for IGF-I in the liver of common carp. However, there was no significant changes in the expression levels of hepatic IGF-I mRNA in GH-treated and saline control fish as indicated by the intensities of the bands. In rainbow trout (*Oncorhychus mykiss*), after intraperitoneal injection of salmon GH at doses of 0.1 or 1.0 μg/g body weight, plasma GH levels increased to a maximun after 6 hr and declined rapidly thereafter (Moriyama, 1995). Therefore, the clearance rate for GH in common carp may also be very fast and the effect of GH on IGF-I expression was diminished during the long chronic injection time courses.

To improve the experimental design, we turned to use a short and acute injection time course to study the expression of hepatic IGF-I. Forty μg of total RNA was fractionated and Northern blot analysis was carried out to measured the hepatic IGF-I mRNA level (Fig. 4.3). However, there was no obvious band observed after autoradiography as compared to the actin positive control. Therefore, the expression level of hepatic IGF-I may be so low that could not be detected using Northern blot analysis of the liver total RNA and RT-PCR was performed to examine the expression of IGF-I mRNA.

Lane 1 -- saline control
Lane 2 -- 100 ng sbGH per g total body wet weight
Lane 3 -- 1,000 ng sbGH per g total body wet weight
Lane 4 -- RNA size marker

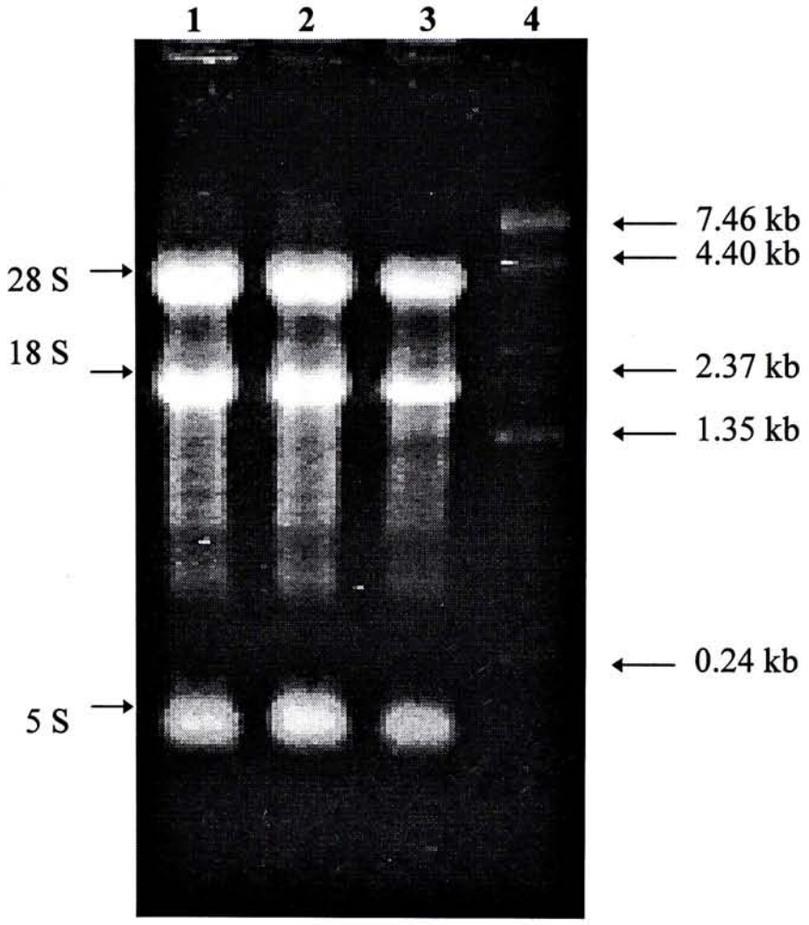

Fig. 4.1 The RNA gel showing the integrity of the pooled RNA samples (10 μg) obtained from injection time course 1 (Section 4.3.1.1).

Lane 1 -- saline control
Lane 2 -- 100 ng sbGH per g total body wet weight
Lane 3 -- 1,000 ng sbGH per g total body wet weight

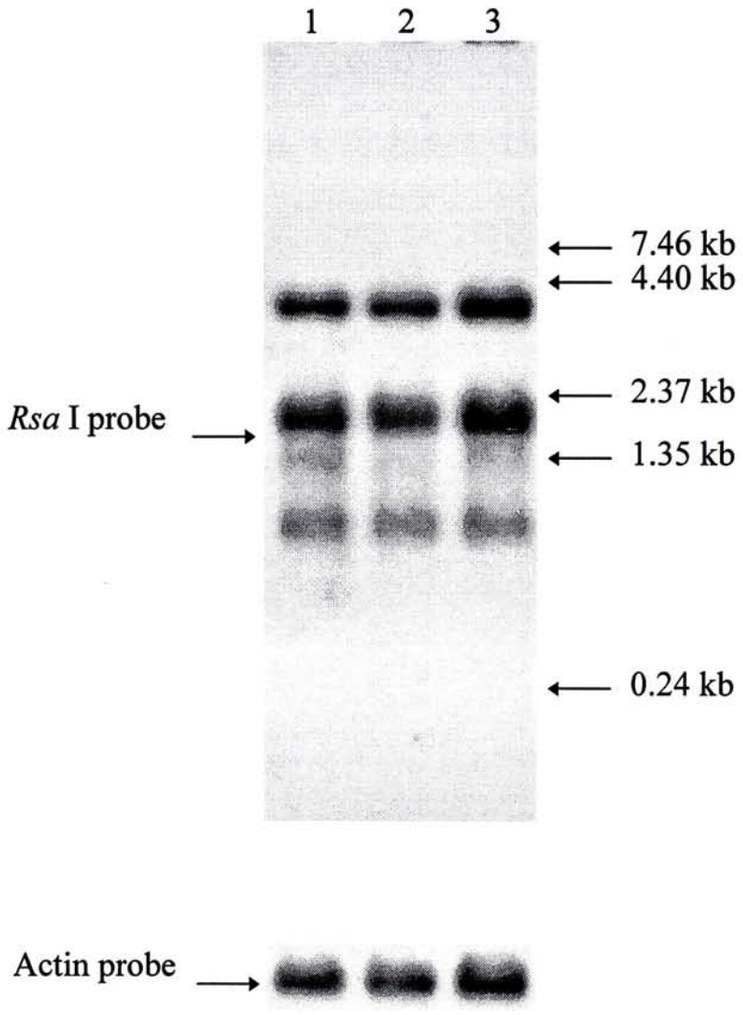

Fig. 4.2 Northern blot analysis of the pooled polyA$^+$ mRNA (10 μg) obtained from injection time course 1 (Section 4.3.1.1). The probes used were: (1) *Rsa* I fragment of common carp IGF-I cDNA (which c6ntains the B, C, A and D domains coding for mature IGF-I peptide); (2) zebrafish actin probe (control).

Lane 1, 2 and 3 -- saline control
Lane 4, 5 and 6 -- 100 ng sbGH per g total body wet weight
Lane 7, 8 and 9 -- 1,000 ng sbGH per g total body wet weight

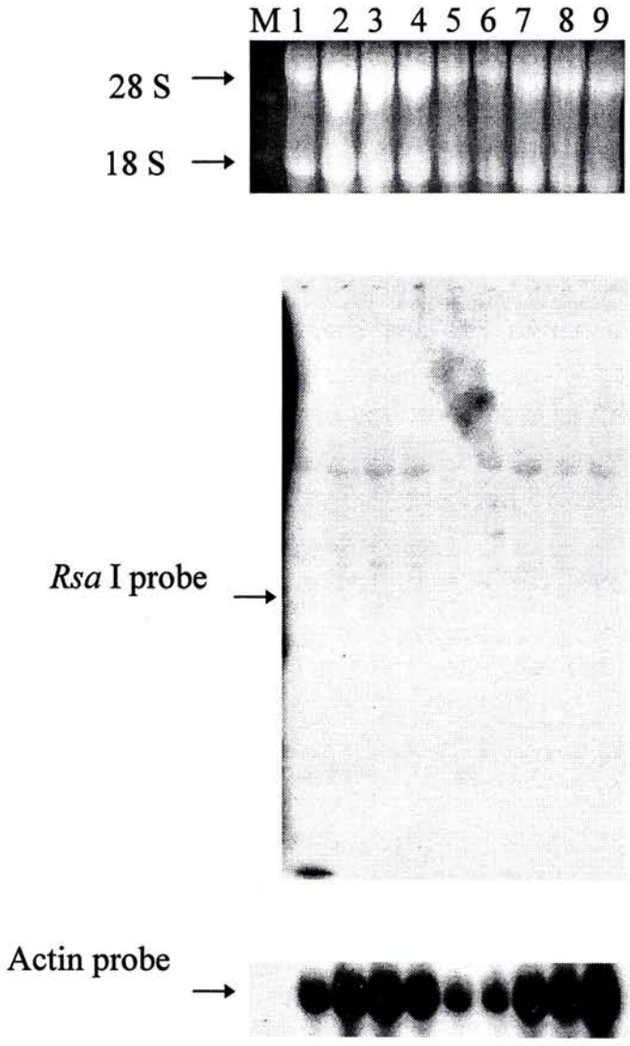

Fig. 4.3 RNA gel and Northern analysis of the individual total RNA samples (40 μg) obtained from injection time course 2 (Section 4.3.1.2). The probes used were: (1) *Rsa* I fragment of common carp IGF-I cDNA (which contains the B, C, A and D domains coding for mature IGF-I peptide); (2) zebrafish actin probe (control). Lane M was the RNA marker.

After RT-PCR, using 1 μg of total RNA as template, the PCR products were fractionated by electrophoresis. (Fig. 4.4). The IGF-I mRNA was amplified as a 480 bp DNA fragment and the intensity of each band was analyzed using a densitometer. Fig. 4.5 shows the relative levels of hepatic IGF-I mRNA normalized by actin control. The level of hepatic IGF-I mRNA was elevated ($P<0.09$) after injection with 100ng/g fGH as compared to saline control. However, the level of hepatic IGF-I mRNA following i.p. injection of 1,000 ng/g sbGH was similar to the saline control.

In rainbow trout, the levels of hepatic IGF-I mRNA increased significantly (~2 to 3 fold) in response to i.p. injection of 10 μg bovine GH at 6 and 12 hr (Shamblott *et al.*, 1995). In *Sparus* liver, Northern analysis revealed that the IGF-I mRNA level increased about fivefold after i.p. injection of human GH at a dose of 1.0 μg/g body weight (Duguay *et al.*, 1996). After i.p. injection of salmon GH at doses of 0.1 or 1.0 μg/g body weight to rainbow trout, plasma IGF-I levels were elevated after 12 hr, reaching maximum (~2 to 3 fold) at 24 hr and (after the higher dose of GH) remaining at 78 hr after GH injection. The effect of GH on plasma IGF-I level was dose-dependent (Moriyama, 1995).

From the above studies, GH treatment can significantly elevated the hepatic IGF-I mRNA level. However, in the present study, we found that the common carp hepatic IGF-I mRNA level (after the lower dose sbGH) was increased by only one-third the saline controls but no significant change was detected in the higher dose of GH. Therefore, the response of common carp to GH was found to be different from other teleosts. To confirm the results obtained in present study, other quantitative methods, such as competitive RT-PCR, Northern blot analysis using PolyA$^+$ mRNA and Ribonuclease protection assay, may be used to study the time course *in vivo*.

Lane 1, 2 and 3 -- saline control
Lane 4, 5 and 6 -- 100 ng sbGH per g total body wet weight
Lane 7, 8 and 9 -- 1,000 ng sbGH per g total body wet weight
Lane 10 -- negative control

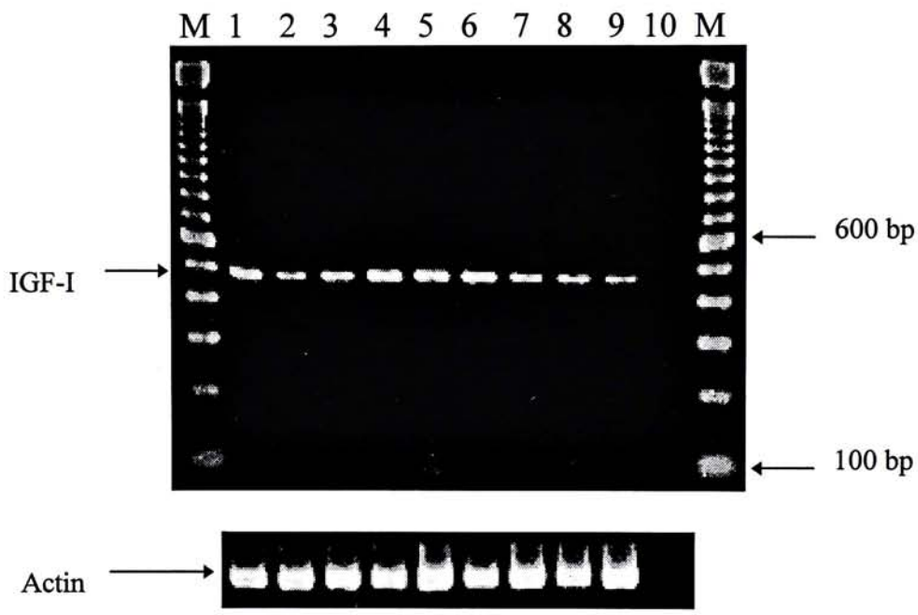

Fig. 4.4 Agarose gel electrophoresis of RT-PCR products using total RNA obtained from time course 2 (Section 4.3.1.2). The gel was stained with EtBr after electrophoresis. Upper panel shows the result of using the common carp IGF-I specific primers. Lower panel shows the result of using the β-actin primers (control). Lane M was the 100 bp marker.

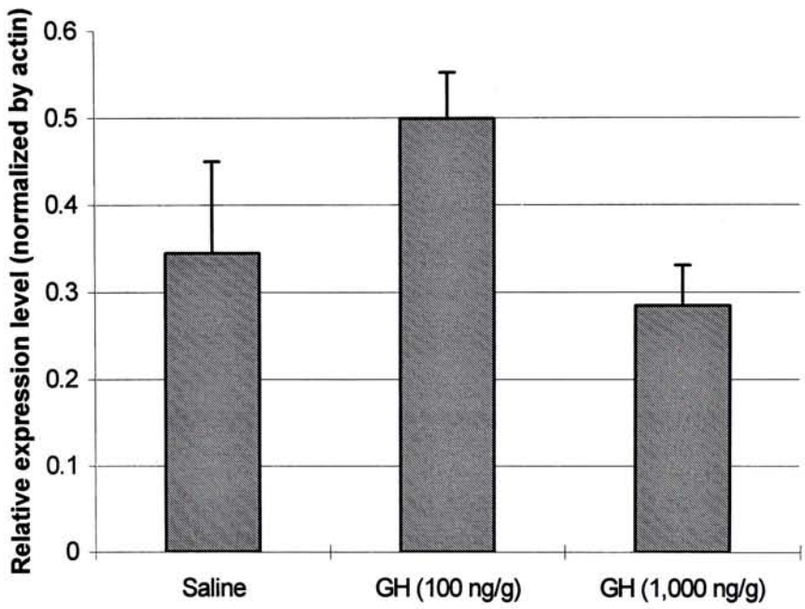

Fig. 4.5 Relative levels of hepatic IGF-I mRNA in control and GH-treated common carp as measured by RT-PCR. Groups of fish (n = 3) received 100 ng and 1,000 ng per g total body wet weight of sbGH or saline vehicle. Each bar with vertical line represents mean ± S.D..

In vitro assay using cultured cells would also be useful to study if GH induces IGF-I in hepatocytes.

Although a feature of the RT-PCR is that it can provide high sensitivity for the detection and amplification of specific nucleic acid sequences, it also presents a substantial problem in attempting to apply to quantitative applications. The impact on the absolute amount of amplified product produced by experimental variations such as pipetting error, initiation of misprimed amplifications, variations in amplification efficiency due to naturally occurring or introduced inhibitors, subtle differences in reagent purity or reaction composition, differences in amplification kinetics related to the number of initation events (particular for low-copy-number specimens), and even minor reaction-to-reaction differences in reaction temperature control for thermal cyclers are all exaggerated by exponential amplification. The net result is that, because of these factors, a consistent relationship between the amount of input target template and absolute amount of amplified product is very difficult to obtain (Krieg, 1996).

To develop approaches to harness the power of the PCR for quantitative applications, the majority of investigators have attempted to provide some form of internal control for the variability that can be expected.

In my present study, IGF-I was coamplified along with actin (heterologous sequence) expected to be present at a relatively constant level as an endogenous component of the sample using distinct primers. However, the use of a heterologous template as an internal control provides only a gross control for overall efficiency of amplification, such as revealing effects that might be expected if a specimen contained a substance that inhibited the PCR. Other inherent differences related to the use of a heterologous sequence, such as differences in template abundance and in primer binding efficiencies for the two templates, render this approach less than ideal.

Recently, another approach of RT-PCR, known as competitive RT-PCR, is used. The target template is coamplified along with increasing known amounts of a closely matched, usually synthetic, template having the same primer binding sequences, whose derived amplified product can be discriminated and differentially quantified relative to the product from the target template. This approach provides more stringent internal control and the multipoint titration effectively provides the equivalent of multiple confirmatory reactions for each sample, minimizing the potential confounding effects of aberrant reactions on accurate quantitation. In addition, extrapolation onto a tiration plot to determine the tiration equivalence point (where the amount of product derived from the target template is equal to the amount of product derived from internal standard) may provide improved accuracy compared to calculations based on single point ratio of the two products, particularly if the absolute amounts of the two products for a particular reaction differ widely and if the reactions are carried to the postexponential phase of amplification. The obvious disadvantage of this multipoint titration approach is the requirement to perform multiple reactions, with corresponding increases in cost and labor-intensiveness (Krieg, 1996).

Other more sensitive and specific methods such as Northern blot analysis of polyA$^+$ mRNA and ribonuclease protection assay may be used to quantify the amounts of mRNA (Shamblott and Chen, 1993; Duguay *et al.*, 1996). The major disadvantage of the above methods is the use of radioactive isotope.

CHAPTER 5 SEARCH FOR IGF-II GENE WITH GENOMIC SOUTHERN BLOT ANALYSIS

5.1 Introduction

Both IGF-I and IGF-II cDNA sequences were identified in seabream and rainbow trout (Shamblott and Chen, 1992; Duguay *et al.*, 1996). Recently, another fish IGF-II cDNA was isolated from tilapia brain cDNA library (Chen *et al.*, 1997). The alignment of seabream and rainbow trout preproIGF-II amino acid sequences revealed that this hormone has also been well conserved among the teleosts The overall amino acid sequence similarity was 84 % and there were only three amino acid differences, all located in the C-domain, between seabream and rainbow trout IGF-II. Even the nucleotide sequence identity had 85% match. The E-domains were also well conserved with 80% similarity (78/98 amino acids). The amino acid sequences of E-domians in human and fish proIGF-II only show 37% identity.

To identify the presence of IGF-I and -II in common carp, we screened a genomic library for IGF genes in the genome of common carp but only identified ten IGF-I clones. Therefore, the presence of other IGF subtypes might not be detectable if the common carp IGF-I cDNA (conserved B, C, A and D domain of mature peptide) probe used in the genomic library screening could only identify IGF-I sequences.

In rainbow trout, the absence of pattern similarity in genomic Southern blot analysis suggested that rainbow trout IGF-I and IGF-II are encoded by two different genes (Shamblott and Chen, 1992). Therefore, the nucleotide sequences of IGF-I and IGF-II in common carp may be quite different. To identify if IGF-II is in the genome of common carp, we used trout IGF-II cDNA (more related to the sequence of common carp IGF-II if present) as a probe for Southern blot analysis of common carp

genomic DNA. Presumably, IGF-II sequence would be able to identity IGF-II of other species.

5.2 Materials

DNA grinding buffer (Section 5.3.1)
0.1M Tris
0.05M Na_2EDTA
0.2M NaCl
1% SDS
10mg/ml Proteinase K (Boehringer Mannheim)

10M Ammonium acetate (Section 5.3.1)
770g ammonium acetate
Add ddH_2O to 1 liter, sterilize by filtration.

50X Tris borate (TBE) (Section 5.3.3)
54g Tris base
27.5g boric acid
20 ml 0.5M EDTA, pH 8.0
Add ddH_2O to 1 liter.

6X Loading buffer for agarose gel electrophoresis (Section 5.3.3 and 5.3.4)
0.25% bromophenol blue
0.25% xylene cyanol FF
40% (w/v) sucrose in water

50X Tris acetate (TAE) (Section 5.3.4)
242g Tris base
57.1 ml glacial acetic acid
100 ml 0.5M EDTA, pH 8.0
Add ddH_2O to 1 liter.

20X SSC (Section 5.3.5)
175.3g NaCl
88.2g sodium citrate
Add ddH_2O to 1 liter, adjust pH to 7.0 with NaOH, sterilize the buffer by autoclaving for 20 min at 15 lb/sq. in liquid cycle

10% SDS (Section 5.3.5)
100g SDS
Add ddH_2O to 1 liter, heat to 68°C, adjust pH to 7.2

5.3 Methods

5.3.1 Preparation of Genomic DNA from Carp Testis

Procedures as described in Section 2.3.1.

5.3.2 Restriction Enzyme Digestion of Genomic DNA

Genomic DNA samples were digested with restriction enzymes. About 20 μg of genomic DNA was digested with excess amount of restriction enzymes. *Bam* HI, *Eco* RI, *Hind* III and *Xba* I were used to digest the genomic DNA in 100 μl of the corresponding buffer. The reaction mixtures were incubated at 37°C overnight. The digested DNA samples were ethanol precipitated and further dissolved in 30 μl sterilized water.

5.3.3 Southern Blotting of the Digested Genomic DNA

Thirty μl of the digested DNA samples were fractionated in a 0.9% agarose gel in TBE buffer (Section 5.2). The fractionated DNA was transferred to Hybond-N nylon membrane after EtBr staining as described in Section 3.3.8.

5.3.4 Preparation of the Trout IGF-II Specific Probe

The trout IGF-II cDNA clone was obtained from Dr. Thomas T. Chen (University of Maryland, Bethesda, U.S.A.). The clone was digested with *Eco* RI and *Xho* I restriction enzymes in an appropriate buffer, releasing the insert from the pBluescript SK vector. After 1.5% agarose gel electrophoresis in 1X TAE buffer (Section 5.2), the DNA fragment was excised and purified using Gene Clean method

as described in Section 2.3.5. The *Eco* RI-*Xho* I fragment released contained the entire coding region for trout IGF-II.

5.3.5 Genomic Southern Hybridization

The zebrafish actin probe (Chan K.M. unpublished work) was used as positive control. The blot was hybidized with the IGF-II probe and then with the actin probe. The probes were labeled with ^{32}P-dCTP using a Nick Translation kit (GibcoBRL) and purified by NucTrap® push columns (Stratagene) as described in Section 3.3.5. The blot was prehybridized at 65°C for 1 hr and then hybridized at 65°C for 2 hr in Rapid-hyb buffer (Amersham). After hybridization, the filter was first washed twice in 2X SSC (Section 5.2) at room temperature for 15 min each, twice in 2X SSC, 0.1% SDS (Section 5.2) at 65°C for 30 min each and finally exposed to Kodak BIOMAX™ MR film at -70°C for 4 days between intensifying screens.

5.4 Results and Discussion

In common carp, genomic Southern blot analysis revealed that two gene fragments hybridized with the IGF-I Ea2 cDNA probe in the genomic DNA digested with *Eco* RI and *Xba* I, whereas *Hind* III digested DNA showed three bands (Liang *et al.*, unpublished data). The intron interrupting the exon 2 and 3 is approximately 10 kb in the chum salmon IGF-I gene (Kavsan *et al.*, 1993), and might contain a site for *Hind* III in the common carp IGF-I gene or IGF-II gene (*Eco* RI, *Hind* III and *Xba* I restriction sites are not present in the cloned common carp IGF-I Ea2 cDNA, i.e. the exons). The result suggested that there are at least 2 IGF genes in the common carp genome.

To identify the presence of both IGF-I and -II in the genome of common carp, we screened a genomic library using the IGF-I cDNA *Rsa* I fragment (containing the conserved B, C, A and D domains) for IGF genes. Presumably, the IGF-I conserved probe would be able to hybridize with both the IGF-I and IGF-II genes. After library screening, only IGF-I gene was identified but no IGF-II gene was found. The two *Xba* I fragments from the positive clones identified with sizes 7 kb and 4 kb are similar to the bands found in Southern blot analysis of the *Xba* I digested common carp genomic DNA using *Rsa* I fragment of the common carp IGF-I cDNA as a probe. These two *Xba* I fragments were found to be contiguously linked together and therefore they could be from the same gene but spilted by an intron that contains an *Xba* I site. For the three *Hind* III gene fragments found in the Southern blot analysis, they are also likely to be linked.

In rainbow trout, IGF-I was found to be only 43.3% nucleotide sequence identical and 60.9% amino acid similar to IGF-II (Shamblott and Chen, 1992).

Therefore, the common carp IGF-I probe used in library screening may only identify IGF-I but not IGF-II.

Mammalian IGF-I and IGF-II are encoded by separate genes. In rainbow trout, IGF-I and IGF-II are also encoded by two separate genes as indicated by the absence of pattern similarity in the genomic Southern blot analysis using either IGF-I or IGF-II probe (Shamblott and Chen, 1992). Therefore, the pattern of genomic Southern blot analysis for IGF-II may be different from IGF-I in common carp.

Then, we used trout IGF-II cDNA as a probe (presumably more similar to carp IGF-II) for Southern analysis of common carp genomic DNA. After hybridization, no obvious bands were detected from the blot as compared to the positive control (Fig. 5.1). The absence of pattern in the blot suggests that (1) the nucleotide sequence of common carp IGF-II may be very different from the trout IGF-II or other fish so that the probe used could not identify IGF-II in common carpIGF-II. It is not likely as the alignment of rainbow trout and seabream which revealed that this hormone is well conserved among teleosts. ; or (2) common carp may not have IGF-II in the genome; or (3) the experiment did not work (?). However, we repeated the experiment for a total of three times, only the rtIGF-II probe did not work but zebrafish actin probe can hybridize to the blots. Therefore, until now, only IGF-I gene but no IGF-II gene could be identified in the common carp genome.

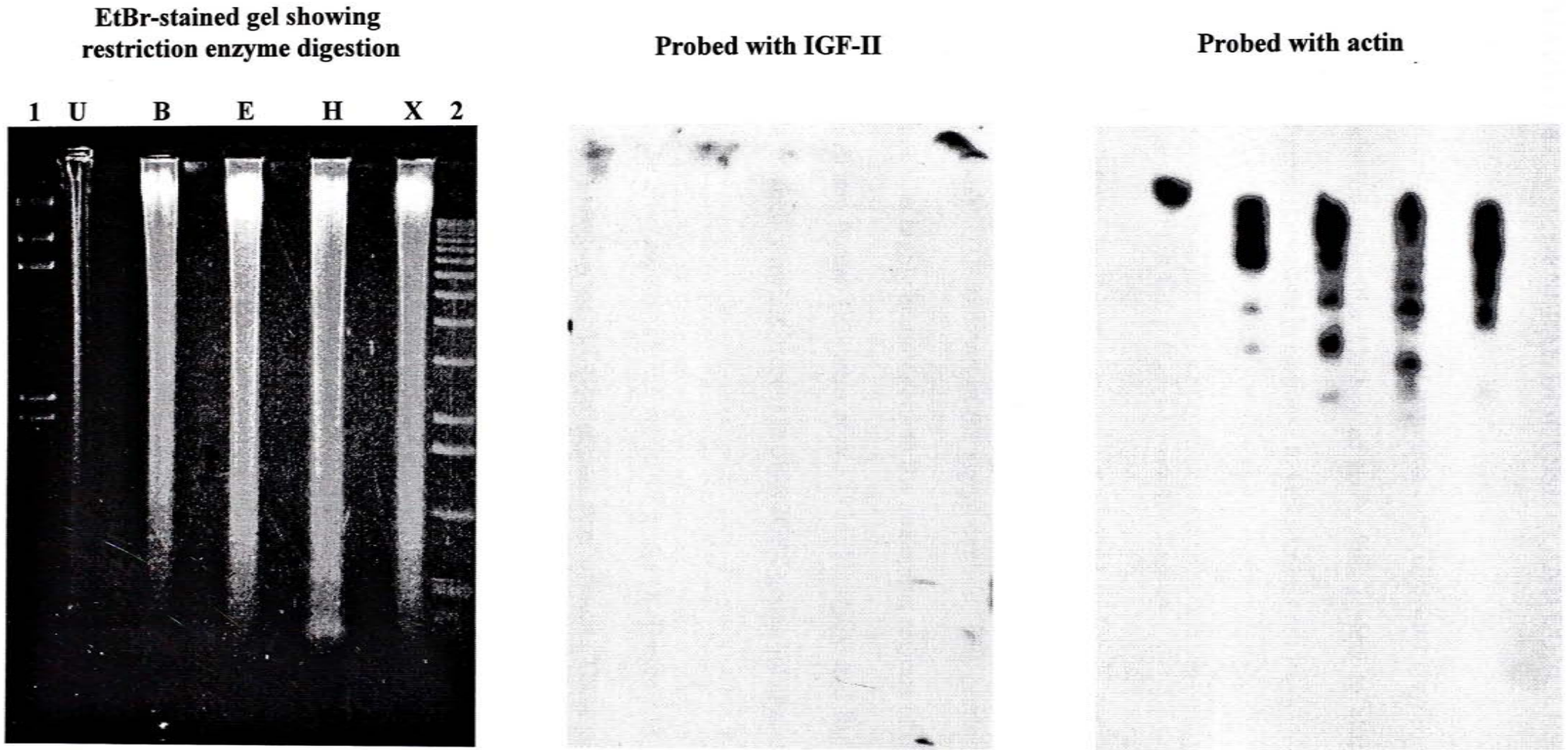

Fig. 5.1 Southern blot analysis of common carp genomic DNA. The probes used were: (1) trout IGF-II cDNA; (2) actin probe as positive control. The corresponding restriction enzymes used in each lane are labeled as U (uncut), B (*Bam* HI), E (*Eco* RI), H (*Hin*d III) and X *(Xba* I). Lane 1 was loaded with the λ *Hin*d III marker, whereas lane 2 was loaded with the l kb marker.

CHAPTER 6 GENERAL DISCUSSION AND CONCLUSION

This thesis reports the use of common carp (*Cyprinus carpio*) as a tropical fish model to study the molecular biology of IGFs with a view to elucidate their gene structure and gene expression in response to GH administration.

Cloning of the Common Carp IGF-I Promoter Region

In the present study, we reported the cloning of the 5' region of IGF-I gene from common carp using genomic PCR. This clone carries an inset of 840 bp encompassing the 5'-UTR and part of the signal peptide. This portion of the common carp IGF-I gene was highly homologous to the analogous regions of other IGF-I genes such as chicken and human (Rotwein *et al.*, 1986; Kajimoto and Rotwein, 1991) if a GAGA-like sequence, which is present in other IGF-I genes but absent in teleost IGF-I genes, is deleted.

As in the chicken, human and rat IGF-I genes (Kajimoto and Rotwein, 1991), no consensus TATA box or AT-rich region was present. These are the promoter elements required for the RNA polymerase to locate the start site in a relative fixed location. As a result, multiple start sites are used in the transcription of IGF-I (Kajimoto and Rotwein, 1991). Like chicken, human and rat, there is a TATA-like consensus sequence approximately 250 bp 5' upstream of the first methionine codon of the common carp IGF-I coding sequence. In contrast, there is no CCAAT-like sequence.

We are now in a position to analyze (1) the promoter activity of this DNA fragment, (2) determine the transcription start sites in the common carp IGF-I gene

and (3) determine the protein factors that interact with the carp IGF-I genes with or without stimulation by GH.

Search for Multiplicity of IGF Gene(s)

The investigation of IGF gene structures in fish would extend the evolutionary picture for these hormones and facilitate our understanding of the features of the IGF genes that are common to all vertebrate species.

The cloned common carp IGF-I gene, which is only about 13 kb in length, appears to be much more compact than the mammalian and avian counterpart. As in other species, however, the mature IGF-I peptide consists of 70 amino acids encoding by exons 2 and 3.

In the present study, we also found that the common carp IGF-I gene is interrupted by four exons with an additional potential splice donor site at the 3' end of E-peptide similar to the chum salmon IGF-I gene (Kavsan *et al.*, 1993). This increases the complexity of IGF-I gene expression and IGF-I biosynthesis in common carp.

In one of the ten isolated genomic clones, P6, the restriction pattern was found to be different from other genomic clones. However, it binds to the common carp conserved IGF-I cDNA probe and specific E-domain probe. Thus, this clone may be a subtype of the IGF-I gene. To confirm the presence of other related IGF-I genes, this clone may be a potential target.

In the search for IGF-II gene, we found that the common carp conserved IGF-I cDNA probe, presumably being able to bind to both IGF-I and IGF-II genes, can only detect IGF-I related genes but not others. It was confirmed by the screening of common carp genomic library and the liver cDNA library that only IGF-I gene or cDNA was found. However, the absence of positive signal in Southern analysis of the

common carp genomic DNA using the entire coding region of trout IGF-II cDNA may indicate the absence of IGF-II gene or the nucleotide sequence is totally different from other species.

IGF-I Gene Expression Studies

From the expression studies, we found that the chronic injection of GH to common carp did not increase but acute injection did slightly increase IGF-I mRNA level. This indicates that the clearance rate of GH might be very fast. In addition, we have to modify the experimental design by increasing the sample size and using juvenile carps of larger size. Because the average weight of the juvenile common carp used is only 4 to 6 g so that the amount of the individual liver tissue is so little for preparing enough polyA$^+$ mRNA for Northern analysis. However, using RT-PCR, we found that the dose of 100 ng/g could increase the IGF-I mRNA level in the liver of common carp.

Conclusions

(1) A DNA fragment of the common carp IGF-I promoter region was obtained by PCR.

(2) A genomic clone of 13 kb was isolated from common carp. This clone carries the entire IGF-I gene which is found to be more compact than other known vertebrate IGF-I genes.

(3) The gene structure of the common carp IGF-I is different from other species in the number of introns.

(4) Using RT-PCR, IGF-I expression in common carp was found to be induced by GH administration.

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